

"DOUBLE STRANDED RIBONUCLEIC ACID  
FROM UNINFECTED RAT TISSUES:  
ISOLATION, PURIFICATION AND CHARACTERIZATION"

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Doctor of Philosophy  
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by

R. PARRY MONCKTON

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I am also grateful to Dr J.F.B. Mercer and Dr R.E.H. Wottenhall for support, and helpful and encouraging discussions.

Statement

This thesis presents an account of research carried out by myself in the Department of Molecular Biology, Research School of Biological Sciences, The Australian National University, Canberra

A thank-you also to all fellow members of the Unit, for their help and good humour. I also thank Mrs Joan Madden for her skill and patience in typing this thesis.

Financial support during the study was provided by a Commonwealth Postgraduate Research Award.

R. P. M. M. M.



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## ABBREVIATIONS

iv.

RNA =	ribonucleic acid
DNA =	deoxyribonucleic acid
dsRNA =	double stranded RNA
nRNA =	nuclear RNA
HnRNA =	heterogeneous nuclear RNA
mRNA =	messenger RNA
rRNA =	ribosomal RNA
tRNA =	transfer RNA
pre before rRNA =	precursor rRNA
tRNA	tRNA
mRNA	mRNA
A =	adenylic acid
G =	guanylylic acid
C =	cytidylic acid
U =	uridylic acid
poly(A) =	a single ribonucleotide strand of adenylic acid
poly(AU) =	a single ribonucleotide strand of repeating adenylic acid - uridylic acid
poly(I).poly(C) =	a synthetic double stranded ribonucleotide strand of inosinic acid and cytidylic acid
oligo -(dT) =	oligo deoxythymidylate

## Buffers and Solutions

TSE =	50mM Tris-HCl, 1mM EDTA and 0.1M NaCl, pH6.9
SSC =	0.15M NaCl, 0.015M NaCitrate, pH7.4
0.1xSSC =	one tenth strength of SSC
2xSSC =	twice strength SSC
high salt =	buffer or solution containing 0.3M NaCl e.g. 2xSSC
low salt =	buffer or solution containing a low concentration of NaCl, 50mM or less e.g. 0.1xSSC

## SUMMARY

A double stranded species of RNA(dsRNA) was discovered in uninfected rat tissues. dsRNA was detected in whole rat liver and testes cells, although the amount is very small. The text describes the detail of experiments concerned with the isolation, purification and characterization of dsRNA. Experiments were designed with the aim of preparing a purified fraction of dsRNA, uncontaminated with single stranded RNA fragments. dsRNA was prepared by ribonuclease (A and T<sub>1</sub>) digestion and further purified by cellulose chromatography. The properties characteristic of a double stranded molecule were extensively investigated to ensure the double stranded nature of the purified material.

dsRNA was prepared from whole cellular, nuclear or cytoplasmic RNAs isolated by sodium dodecyl sulphate-phenol extractions of whole liver or testes cells, liver nuclei or liver cytoplasm, respectively. dsRNA was prepared free of single stranded RNA by digestion with specific ribonucleases. Optimum conditions for ribonuclease treatment, i.e. the enzyme concentration and enzyme:RNA ratio etc., were carefully investigated for the preparation of intact dsRNA. The ribonuclease resistant RNA obtained after digestion was further purified by stepwise elution of a cellulose column. Using this technique of eluting with three mixtures of buffer and ethanol in increasing ratio, a purified fraction of dsRNA free of RNA with single stranded tails, was found in the third eluted fraction. The chemical and physical properties of the purified material were examined and confirmed the double



stranded nature of the material, but excluded the possibility of a RNA-DNA hybrid. The properties used to investigate the double stranded nature, and which could be demonstrated for the purified fraction of dsRNA were as follows:-

- (1) Ribonuclease resistance. This property was employed in the preparation of dsRNA, although it does not exclude the possibility of the resistance of single stranded ribopolymers e.g. Poly(A).
- (2) Heat denaturation profile. A biphasic heat denaturation profile with a transition from ribonuclease resistance to ribonuclease sensitivity at a sharply defined denaturation temperature in high salt concentrations.
- (3) Base analysis.  $A = U$ ,  $G = C$ , signifying base paired character.
- (4) A reaction to an antiserum prepared specifically against a synthetic dsRNA, poly(I).poly(C). Rat liver and testes dsRNA react to this antiserum, but not when heat denatured.

Rat liver cellular dsRNA revealed two discrete sizes on gel electrophoretograms. Rough calibration of the gels with purified cellular RNA and tRNA, revealed that the slower moving species is around 60 nucleotide pairs in length.

Evidence is presented that the majority of dsRNA is of nuclear origin, being derived from HnRNA but not ribosomal or pre-ribosomal RNA or tRNA. Purified rat liver nuclear RNA was fractionated and dsRNA was isolated from the higher molecular weight fractions (>45s) of the HnRNA as well as lower molecular weight fractions. "Snap-back" analysis

revealed that dsRNA isolated from this high molecular weight fraction, was derived from close "hairpin" loops in the HnRNA structure. Looped regions in the HnRNA may in fact contribute the majority of dsRNA isolated from the cell.

Poly(A) segments were found co-chromatographing with dsRNA, during cellulose chromatographic separation. The dsRNA and poly(A) did not appear to be linked.

The possible functions of dsRNA are discussed in detail. In particular, the relevance of the close "hairpin" loop origin for dsRNA is discussed in relation to the hypothesis of mRNA being processed from HnRNA. Other functions of dsRNA are also considered, including possible involvement in interferon induction, in the regulating mechanism of protein synthesis, and in the self duplication of the message.

#### 1-4 THE PHYSICAL AND CHEMICAL PROPERTIES OF A DOUBLE STRANDED MOLECULE

##### 1-4-1 Ribonuclease Resistance

##### 1-4-2 Solubility

##### 1-4-3 Buoyant Density

##### 1-4-4 Lipid Composition

##### 1-4-5 Denaturation and Renaturation

##### 1-4-6 Reaction to Specific Enzymes

##### 1-4-7 Characteristic Behavior on Column Chromatography

#### 1-5 OTHER PHYSICAL PROPERTIES WHICH ARE USEFUL IN STUDYING dsRNA

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CHAPTER I - GENERAL INTRODUCTION1-1 DOUBLE STRANDED RNA IN VIRUSES - THE REPLICATIVE FORM

Current concepts of molecular biology, dictate that the function of the two nucleic acids are clearly separated: DNA is the depository of genetic information, RNA is involved in the expression of this information, either at the transcriptional level of DNA, or in the translation process of messages into proteins. DNA is a self replicating molecule which exists normally as a double helix. RNA is synthesized along a DNA strand and is usually single stranded.

It would appear, however, that there are exceptions to this rule. Immediately viral RNA comes to mind; it can perform both functions in the host cell, it possesses the ability to replicate and to be translated. It was first demonstrated that the RNA component of viruses, plant and animal was infectious, and hence carried its own genetic information (GIERER and SCHRAMM, 1956; COLTER, BIRD and BROWN, 1957; DAVIS, STRAUSS and SINSHEIMER, 1961). Later it was demonstrated that viral RNA replication was not dependent on the integrity of host cell DNA and did not require its transcription or the synthesis of new DNA (REICH and FRANKLIN, 1961; REICH *et al.*, 1962; SIMON, 1961). This picture may be a little over-simplified in view of the activity of reverse transcriptase in oncogenic viruses and integrated viral DNA. Generally, however, viral RNA is independent of the host cell machinery. Complexes of RNA

replicase bound to an RNA template were found in cells infected with animal viruses (BALTIMORE and FRANKLIN, 1962) and bacteriophages (WEISSMAN, SIMON and OCHOA, 1963). In the MS2 and Q $\beta$  phage systems, the free enzymes were isolated, and were found to have a preference for homologous viral RNA template. These discoveries meant that viral RNA could be a self replicating polycistronic messenger, and the next manifestation would be the discovery of the double stranded base-paired replicative form (R.F.).

These double stranded forms were discovered in cells infected with picorna viruses (MONTAGNIER and SANDERS 1963; BALTIMORE, BECKER and DARNELL, 1964) plant viruses (SHIPP and HASELKORN, 1964; MANDEL *et al.*, 1964) and phages (KELLY and SINSHEIMER, 1964; WEISSMANN *et al.*, 1964). Virus specific double stranded RNA (dsRNA) has been found in practically all replicating virus systems (except oncogenic RNA viruses) and several double stranded RNA viruses, e.g. reovirus are known to exist. dsRNA has been established as the replicative form of practically all RNA viruses.

#### 1-2 DOUBLE STRANDED RNA IN "UNINFECTED CELLS", EARLY VIRUS STUDIES AND INTERFERON INDUCTION

Research into the replicative forms of viruses and the mechanism of interferon induction, led to the discovery of dsRNA in "uninfected" animal cells. The anti-viral agent, interferon, has been found to be induced by many different RNA and DNA viruses (HO, 1966) although the mechanism of induction is not well understood. Based on the findings that



material from  
 a variety of sources induced interferon (LAMPSON *et al.*, 1967; TYTELL *et al.*, 1967; and FIELD *et al.*, 1968) TYTELL and co-workers speculated that the double stranded replicative form of viral RNA was responsible for the interferon induction. However, COLBY and CHAMBERLIN (1969) and COLBY and DUESBERG (1969) in investigating the hypothesis that the virus specific dsRNA may be the inducer of interferon in cells infected with vaccinia virus, made reference to the discovery of a dsRNA in the "uninfected" as well as the infected cells. It is known that interferon can be induced in conditions under which little or no viral multiplication occurs, and that interferon could be induced by synthetic, but not viral, double stranded ribopolymers. These observations suggested that dsRNA of "uninfected" cells could be an inducer of interferon (KIMBALL and DUESBERG, 1971). In fact, there have been reports suggesting the presence of dsRNA in "uninfected" cells. STERN and FRIEDMAN (1970) discovered the presence of a double stranded form of RNA synthesized in the presence of actinomycin D in Burkitt lymphoma cells. Using a micro complement fixation by specific anti-dsRNA sera, small amounts of antigen were detected in "uninfected" BHK and KB cells (STOLLAR AND STOLLAR, 1970).

### 1-3 FURTHER REPORTS OF dsRNA PREPARED FROM "UNINFECTED" ORGANISMS - POSSIBLE INVOLVEMENT IN NORMAL CELLULAR METABOLISM

A more important recent finding was that a crude fraction of ribonuclease resistant dsRNA could be isolated from the

normal whole cells of rat liver (MONTAGNIER, 1968; HAREL and MONTAGNIER, 1971). MONTAGNIER, (1968) in fact reported these duplex molecules as being present in ascites cells, chick embryo fibroblasts, and BHK cells. There have been further investigations of dsRNA arising from sources of "uninfected" cells. STERN and FRIEDMAN, (1971) extended their studies and investigated the properties of a ribonuclease resistant RNA isolated from Burkitt's lymphoma, chick liver, and chick embryo fibroblasts. Independent reports have confirmed the presence of a similar ribonuclease resistant RNA from sea urchin embryos (KRONENBERG and HUMPHREYS, 1972) and also from Hela cells (JELINEK and DARNELL, 1972).

The existence of dsRNA in "uninfected" cells was unveiled partly by accident, and from a desire to isolate the interferon inducer. MONTAGNIER's crude dsRNA from rat liver can induce interferon (DeMAEYER, DeMAEYER-GUIGNARD and MONTAGNIER, 1971) but this is not the sole function of dsRNA. It is evident that dsRNA could play other important roles in cellular metabolism. Indeed, we can logically ask two questions. What is the origin of dsRNA within the normal cell? Having answered this, what is its true function(s)?

#### 1-4 THE PHYSICAL AND CHEMICAL PROPERTIES OF A DOUBLE STRANDED MOLECULE

Before attempting to answer the question of origin, and the related biological problems, it was necessary to examine all the real physical and chemical properties of dsRNA that characterize this molecule. Preparations of dsRNA material

reported so far have been crude. Consequently, the purification of a dsRNA fraction was certainly required before furthering the investigations of the function(s) of dsRNA. The importance of establishing the fact that any observed biological effects are due to a truly double stranded molecule cannot be overstressed. Much residual confusion in the literature can be attributed to data derived from the study of inadequately purified material, and to the failure to employ rigorously the physico-chemical criteria of secondary structure which defined dsRNA, before attempting to answer fundamental biological questions. A brief resumé of the physical and chemical properties of a dsRNA molecule will illustrate the methods which are available to detect dsRNA and separate dsRNA from single stranded RNA.

#### 1-4-1 *Ribonuclease Resistance*

The recognition that polyribonucleotide chains involved in extensive hydrogen bonded structures are resistant to endonucleolytic cleavage by pancreatic ribonuclease A (or ribonuclease T<sub>1</sub>), is essential to the investigation of dsRNA. This ribonuclease resistance is quite sensitive to ionic environment (BISHOP *et al.*, 1965; WEISSMANN and BORST, 1963), and is completely abolished at low concentrations of divalent cation and/or NaCl. This resistance is not absolute under any circumstances. Sufficiently high concentrations of enzyme will eventually degrade the molecule, even in high concentrations of NaCl, (BISHOP and LEVINTOW, 1971). The exact nature of this enzymatic attack is not known, but it can be shown by



denaturation of enzyme-treated duplex that single breaks are inserted in the absence of appreciable degradation of the mature molecule (ERIKSON and ERIKSON, 1967).

Preparation of dsRNA in most references heretofore mentioned, involved the removal of single stranded RNA in double stranded preparations with ribonucleases. Although the ribonuclease resistance of dsRNA may be only relative, this method is the only one practically applicable at present. dsRNA has been shown to represent only a very small proportion of the total RNA of the cell (less than 1%) and consequently removal of single stranded RNA is extremely difficult by any other method. In this work, dsRNA was prepared by digestion with ribonucleases. The characteristic properties of a dsRNA molecule are as follows:

#### 1-4-2 *Solubility*

dsRNA is soluble in high NaCl concentration ( $> 1\text{M}$ ) whereas the high molecular weight single-stranded RNA's (~~except tRNA~~) are insoluble. This property can be used to fractionate dsRNA using NaCl (MONTAGNIER and SANDERS, 1966) or LiCl (BALTIMORE, 1966) from the balance of single stranded RNA.

#### 1-4-3 *Buoyant Density*

The buoyant density of dsRNA is lower than that of single stranded molecules in salt solution, due to the greater extent of hydration of the double helix. Hence measurement of buoyant density in equilibrium density gradients of caesium salt has provided a valuable method of identifying dsRNA. Because of the apparent high buoyant density of RNA in ~~salt including~~ CsCl,  $\text{Cs}_2\text{SO}_4$  gradients must be used to separate double and single stranded RNA. The use of  $\text{Cs}_2\text{SO}_4$ , however, complicates

molecular weight determination because of its tendency to cause aggregation and co-precipitation of single stranded RNA. This can cause trapping of dsRNA in precipitated bands of single stranded RNA. This method is unsuitable for large scale dsRNA preparation. The buoyant density of dsRNA is intermediate between single stranded RNA and DNA.

#### 1-4-4 Nucleotide Composition

Double helical, nucleotide paired RNA should contain equivalent amounts of complementary nucleotides ( $A = U$ ;  $G = C$ ). The base <sup>composition</sup>~~sequence~~ can be determined by alkaline hydrolysis and chromatography. The finding of equal values of A and U, G and C constitutes important evidence for the confirmation of a base paired structure. The  $A + U / G + C$  ratio is also related to another important property: the denaturation temperature ( $T_m$ ).

#### 1-4-5 Denaturation and Reannealing

Double stranded nucleic acids undergo transition from ordered helical form to a random coil structure when heated above a critical temperature. This transition (or denaturation) is characterized by an abrupt increase in absorbance at 260 nm. In the case of dsRNA, denaturation is also detected as a transition from ribonuclease resistance to ribonuclease sensitivity, (GEIDUSECHEK *et al.*, 1962) a convenient alternative, as it requires much less material than optical measurements. The  $T_m$  for a particular species of nucleic acid is determined primarily by nucleotide composition and secondarily by the ionic environment in which the measurement is made (DOTY *et al.*, 1959; MARMUR and DOTY, 1962).

In general the  $T_m$  of RNA is substantially higher (ca  $15^\circ$ ) than that of DNA with the equivalent nucleotide composition, secondary structure and molecular weight (BILLETER *et al.*, 1966; BISHOP and KOCH, 1967; GOMATOS and TAMM, 1963). Denaturation of dsRNA results in complete separation of component strands, and is accompanied by a shift in buoyant density. Changes in the electrophoretic mobility can also be used, but in denaturation the occurrence of chain breaks during manipulation must be prevented. Polyribonucleotides are considerably <sup>less</sup> ~~more~~ sensitive to thermal hydrolysis of phospho-diester chain links, yet require higher temperatures for denaturation <sup>than deoxyribopolynucleotides.</sup> Contamination with residual ribonuclease must also be avoided.

At a suitable temperature and salt concentration, the component single strands of dsRNA are capable of reassociation. In practice, however, this is much more difficult to demonstrate. With double stranded viral RNA (RF) only 20% reassociation has been demonstrated (IGLEWSKI and FRANKLIN, 1967; BISHOP and LEVINTOW, 1971). Reassociation does occur, however, and perfectly reformed double helices have been demonstrated in reovirus dsRNA. (WATANABE *et al.*, 1968). Of course renaturation requires optimum temperature and ionic conditions. As with denaturation, high temperatures produce strand scission, and the annealing is hence rendered more difficult.



#### 1-4-6 *Reaction to Specific Antigen<sup>Sera</sup>*

SCHWARTZ and STOLLAR, (1969); STOLLAR, (1970); SCHUR and MONROE, (1969) reported the preparation of antisera capable of specifically detecting dsRNA. It has been shown that antibodies elicited in response to a complex of synthetic double stranded polyribonucleotides and methylated bovine serum albumin (MBSA) bind specifically with multi-stranded structures containing polyribonucleotides, but do not react with double stranded DNA, or natural single stranded RNA. This observation has been utilized in studying the dsRNA of animal cells infected with RNA viruses (STOLLAR and STOLLAR, 1970, 1970). Antiserum prepared against synthetic double stranded polymer, poly(I).poly(C), has been successfully used in the immunochemical detection of dsRNA in leaves of sugar cane infected with Fiji disease virus (FRANCKI and JACKSON, 1972). This antiserum specific to poly(I).poly(C) has been used to detect dsRNA from two different rat tissues and will be described in the context of this thesis. The technique of detecting dsRNA is relatively simple and it affords an extra method of detection of small quantities of dsRNA, or where the dsRNA cannot be easily labelled.

#### 1-4-7 *Characteristic Behaviour on Column Chromatography*

The profile of dsRNA obtained using specified chromatographic separation can be indicative of a dsRNA molecule, as well as a method for purifying crude fractions of dsRNA. In particular, behaviour characteristic of a dsRNA species is obtained when either cellulose or hydroxyapatite columns

are used for separation. These two types of column materials possess the following special property: Separation of dsRNA from contaminating single stranded RNA fragments is achieved due to the double stranded conformation, i.e. the actual base-paired structure. This is in contrast to the alternative molecule<sup>or</sup> sieve methods (Sephadex, agarose, acrylamide, etc.) which separate molecules on the basis of their molecular weight. The cellulose technique will be described in detail in Chapter II of this thesis, but briefly consists of a stepwise elution of a column of unsubstituted cellulose. The technique was originally described by BARBER, (1966) and FRANKLIN, (1966) and was used to separate double stranded replicative forms of viral RNA. This technique is adopted for use on dsRNA in the experiments in this thesis. It is characteristic of dsRNA to chromatograph solely in the third peak after stepwise elution with three buffers of changing concentration (see Chapter II for details).

The hydroxyapatite method was reported first by BERNARDI, (1969). The principle of separation is based on the fact that single stranded polynucleotides are eluted from a column of hydroxyapatite crystals at lower phosphate molarities than rigid double stranded polynucleotides. ~~Using a gradient of changing phosphate molarities, single and double stranded RNAs should elute at different phosphate molarities.~~ This <sup>phenomenon</sup> ~~behaviour~~ therefore similarly allows the detection of characteristic dsRNA behaviour. However, the technique has a disadvantage in purifying dsRNA and will be

referred to in greater detail in Chapter II.

~~Section 1-4 gave a brief outline of the physical and chemical properties of dsRNA. As already stated, in attempting to explain the function of dsRNA within the cell, or its effects on other cells, it must first be positively established that the encumbent material is double stranded. This can be done by applying one or all of the above criteria and determining whether the material meets these requirements.~~

#### 1-5 OTHER PHYSICAL PROPERTIES WHICH ARE USEFUL IN STUDYING dsRNA

The annealing process <sup>can be used as a tool</sup> ~~is beneficial~~ from another point of view. Denatured dsRNA (i.e. single strands) can be annealed to other types of RNA or DNA, from either heterologous or homologous sources. This <sup>is</sup> ~~appears to be~~ an excellent method for illustrating whether dsRNA is of contaminating viral origin. It also affords a method of studying the homology <sup>among</sup> ~~between~~ internal RNA sources. In this way fairly precise decisions can be made as to the origins of dsRNA within the cell. For example, if homology can be demonstrated between dsRNA and mRNA and heterogeneous nRNA, then it is a fairly likely conclusion that double stranded sequences have been inherited by the mRNA in the cytoplasm from nuclear RNA. This point will be discussed in greater detail in Chapter VII.

#### 1-6 FURTHER REPORTS OF dsRNA FROM UNINFECTED CELLS INVOLVING DETAILS OF STRUCTURES AND PROPERTIES OF THE MOLECULE

During the progress of the work detailed in this thesis,



other reports have appeared from workers similarly attempting to isolate and understand the function of dsRNA. A brief resumé of these reports will be given here to enable understanding of perspectives of dsRNA properties and function discussed later in this thesis.

Reports of the properties of dsRNA isolated from "uninfected" cells have been conflicting. Early discoveries of dsRNA in "uninfected" cells were made in parallel to studies of infected cells. COLBY and DUESBERG, (1969) working with vaccinia virus infected chick cells, reported their "uninfected" chick cells contained a ribonuclease resistant RNA which was capable of hybridization to 3% of RNA from viral sources. In a later publication, the properties of the cellular ribonuclease resistant RNA were detailed (KIMBALL and DUESBERG, 1971). In this study they used labelled cultures of rabbit kidney, chick embryo and Hela cells. The method of preparation of the material was to remove contaminating single stranded RNA with ribonucleases A and  $T_1$ . The small fraction of ribonuclease resistant RNA isolated (between 0.23 and 0.45% of total cellular RNA extracted), behaved in a heterodisperse fashion on sucrose gradients (4-20S) with peaks at 7S for rabbit kidney, 9S for chicken embryo, and 11S for Hela cell material. A denaturation temperature of 86°C was obtained. Only the 8-20S material was interferon inducing, and the synthesis of the RNA was 90% inhibited by actinomycin D. This inhibition would suggest a dependence on a DNA template, and it is possible therefore from this and other evidence,

that the majority of their material could have been derived from DNA viruses contaminating the cell cultures.

The investigation of  $T_4$  phage infected *E. coli* (JURALE, KATES and COLBY, 1970) was fairly limited. They reported a very heterodisperse form, 4-16S on sucrose gradients. The ribonuclease resistant RNA (0.6-1% of total RNA), did not give a sharp melting temperature. They reported a 12% hybridization to  $T_4$  DNA and hence suggested that the ribonuclease resistant RNA was a duplex form arising from the overlapping of transcription of complementary DNA strands.

STERN and FRIEDMAN (1970, 1971) investigated dsRNA synthesized in the presence of actinomycin D in "uninfected" cells. The material investigated was primarily isolated from cultured Burkitt's lymphoma, but isolation of similar material was accomplished from human lymphocytes, chick cells, and rat tissues. [ $^3\text{H}$ ] uridine was incorporated into RNA in actinomycin D treated cells at a rate of 0.8-2.3% of that in untreated cells. Of this RNA, 40-68% was found to be resistant to ribonucleases A and  $T_1$ . The ribonuclease resistant RNA from Burkitt's lymphoma showed a heterodisperse pattern of 4-14S on sucrose gradients with a peak at 7S. The nucleotide composition of  $^{32}\text{P}$ -labelled chick embryo fibroblasts, however, does not suggest a simple base paired structure of the material. The behaviour of the ribonuclease resistant core, as STERN and FRIEDMAN, (1971) termed it, tended to confirm this. When denatured over a temperature range, a  $T_m$  of  $70^\circ\text{C}$  was obtained, however only 40% of the material became sensitive to ribonuclease even at  $100^\circ\text{C}$ . No

explanation of this was offered, but it is difficult to reconcile the results with these fractions with the investigations of other workers. It is possible that the conditions for removal of protein were inadequate: no protease was used, but the core was apparently completely digested under conditions of alkaline hydrolysis. It is, of course, possible that this was a different type of dsRNA. In their later paper (STERN and FRIEDMAN, 1971) emphasised that the ribonuclease resistant core was a powerful interferon inducer, and that their material was not a DNA-RNA hybrid. The primary material used in the studies of STERN and FRIEDMAN (1970, 1971) was derived from Burkitt's lymphoma. Since the disease Burkitt's lymphoma itself appears to be cancer mediated by a virus, use of lymphoma cells as a source of dsRNA would cause confliction and abnormalities in the interpretation of their results.

The first mention of dsRNA in "uninfected" cells was by MONTAGNIER in 1968. He extended these studies and described additional work in 1971 (HAREL and MONTAGNIER, 1971). The material from which he isolated his ribonuclease resistant RNA was rat liver. He discovered that it represented a small fraction (0.1-1%) of the total RNA synthesized. This crude fraction of ribonuclease resistant RNA, <sup>when chromatographed on Sephadex</sup> ~~had a sedimentation~~ <sup>showed a pattern</sup> ~~pattern on Sephadex~~ which indicated the heterogeneous nature of the material, 4-18S, with a peak at 8S. The denaturation temperature indicated was 75-77°C at low salt concentrations and the fraction isolated was capable of a 20% hybridization



to rat liver DNA. This ribonuclease resistant RNA was capable of inducing interferon as has already been mentioned (DeMAEYER, DeMAEYER-GUIGNARD and MONTAGNIER, 1971).

A double stranded fraction has also been isolated from sea urchin embryos (KRONENBERG and HUMPHREYS, 1972). This source is especially interesting as it is phylogenically different from the ~~normal tissue~~ cultured <sup>mammalian</sup> cells, which might suggest that dsRNA is a feature of cellular metabolism even at the earliest stages of differentiation. The method of preparation was similar to that used by other investigators, i.e., degradation of the single RNA strands with ribonucleases A and T<sub>1</sub>. However, their investigation of the physical and chemical properties was fairly extensive. They describe the isolation of a small ribonuclease resistant fraction (0.06-0.16%) of RNA which behaves as one would expect for a double helical structure. ~~It shows a heterogeneous dispersion on sucrose gradients~~ <sup>It shows a heterogeneous dispersion on sucrose gradients</sup> ~~It sediments in the 5S region on sucrose~~ <sup>(4-8S) with a peak at 5S.</sup> ~~gradients, but is heterogeneous (4-8S).~~ Its denaturation temperature is 76°C at low salt concentrations, and has a nucleotide composition which ~~reflects a base paired structure,~~ <sup>shows an</sup> ~~except for the~~ excess of adenylic acid residues.

Recent papers have reported nuclear dsRNA derived from nuclei (JELINEK and DARNELL, 1972; RYSKOV *et al.*, 1973; PATNAIK and TAYLOR, 1973). JELINEK and DARNELL, (1972) described the isolation of a ribonuclease resistant dsRNA from Hela cells and Hela cell nuclei. They reported a broad peak of 4S on sucrose gradients and a buoyant density of 1.59. The ribonuclease resistant fraction appears to be approximately

3% of the nuclear RNA. Denatured material is capable of hybridization to 50% of DNA isolated from the same source in 6 h.

RYSKOV *et al.*, (1973) reported the isolation of ribonuclease resistant double stranded material from Ehrlich ascites tumours and cell nuclei. They detailed the presence of two types of dsRNA with long and short chain sequences. These two types were reported as running slightly before and slightly after a 4S tRNA marker on G-75 Sephadex columns; the actual size is not given, although the short sequence would be 10-15 nucleotides in length. They also reported rapid hybridization of dsRNA to DNA.

The most recent study was by PATNAIK and TAYLOR, (1973) working on Ehrlich ascites, sarcoma 180, and L cells. In some experiments the cells were infected with viruses. The results are similar to those of KIMBALL and DUESBERG, (1971). They report the double stranded species are sensitive to actinomycin D and appear to be nuclear in origin, although no nuclei were prepared from whole cells and examined. ~~The double stranded species behaves in a polydisperse fashion on sucrose gradients, with a wide range reported size is 4-13S; polydisperse on sucrose gradients.~~ *of sedimentation.*

In briefly summarizing the work which has been done on ribonuclease resistant dsRNAs, I have not attempted to illustrate the many differences which prevail in the preparation of these RNAs. Differences in the methods of preparation will make obvious differences in physical and/or chemical measurements. Some discrepancies are difficult to explain, for example, there seems to be some variability in

the size of the molecule reported, even when isolated from the same source and using the same method. Sucrose density gradient patterns suggest the size varies over a wide range. It is likely that in some circumstances inadequate digestive conditions may have prevailed, or the true double stranded material had been inadequately separated from contaminating single strands of digestive products. In many cases the use of other techniques of analysis, e.g. gel electrophoresis, to determine size, may have yielded better measurements.

There are also discrepancies in measurements which could not have been realized. ~~Early measurement of~~ <sup>High values for adenylic acid residues in the analysis of base composition were</sup> ~~nucleotide compositions did not realize the fact of high~~ ~~adenylic acid residues.~~ This was probably not commented upon, because ~~the discovery of~~ poly(A) sequences in mRNA and HnRNA were not discovered until recently (MENDECKI, LEE and BRAWERMAN, 1972; RYSKOV *et al.*, 1972; LaTORRE and PERRY, 1973 and references therein).

A larger part of the aim of the experiments described in this thesis, was to attempt to clarify the pure preparation of a dsRNA, having particular regard to the details of physical and chemical properties, the optimum ribonuclease concentrations and the correct ionic conditions. No <sup>extensive</sup> ~~consistent~~ study of dsRNA had been undertaken, and in most reports of dsRNA, little was done to investigate the basic properties characteristic of dsRNA. Similarly, there has been no attempt made at defining the ribonuclease



digestion conditions, a vitally important point if the implications of excess ribonuclease treatment are considered, i.e. the breakage of the dsRNA chain. As previously stated, ribonucleases have a capacity to digest dsRNA depending on the concentration of ribonuclease and the conditions of digestion. It was therefore most important to establish not only the ribonuclease concentration, but also the ribonuclease:RNA ratio in the digestion mixture before attempting to investigate the properties of dsRNA from uninfected rat tissues and to interpret the results. It is obvious that provided careful attempts are made to investigate the physico-chemical properties of dsRNA prepared under defined conditions, valuable conclusions about biological origin and function can be made.

## 1-7 MAJOR QUESTIONS CONCERNING ORIGIN AND FUNCTION OF dsRNA

### 1-7-1 *A Latent Viral Origin*

It could firstly be argued that dsRNA may have a latent viral origin. This is however, unlikely, for the following reasons: dsRNA can be isolated from varied and diverse sources. It arises not only from rat liver and rat testes cells, which are unlikely to both be infected, but even from sea urchin embryos (KRONENBERG and HUMPHREYS, 1972) a most unlikely host for virus infection. Furthermore, the homology with DNA, HnRNA, mRNA regions of HnRNA and mRNA (described in Chapter VII of this thesis) render the possibility more unlikely. It is therefore possible that dsRNA reflected some

heretofore unnoticed function in cellular RNA metabolism. The investigation of dsRNA, as a separate entity in the cell, requires suggestions of origin. Hence other likely sources within the cell are as follows:

#### 1-7-2 *Association of Overlapping Transcripts*

Both strands of the cellular chromosomal DNA might be transcribed at some sites. Alternatively complementary sequences may be present on the same strand. In either case transcription of the DNA would produce complementary single stranded RNAs. The formation of a double stranded RNA may then result from association of the complementary sequences during the extraction process. It has been suggested that dsRNA arises from overlapping transcription of anti polar genes in phage systems (BØVRE and SZYBALSKI, 1969; JURALE *et al.*, 1970). HAREL and MONTAGNIER, (1971) reported hybridization of dsRNA to rat liver DNA; they suggested that the dsRNA is homologous to highly reiterated DNA sequences. There is then the possibility that the double stranded conformation is produced via <sup>double strand formation by transcription</sup> ~~transcriptional annealing~~ in the cell of these complementary highly redundant sequences.

#### 1-7-3 *dsRNA: Part of a Larger Molecule?*

dsRNA may exist in the duplex form as part of another kind of RNA. Double stranded regions of ribosomal RNA, (FRESCO *et al.*, 1960; GOULD and SIMPKINS, 1969; COX, 1970) and tRNA (HOLLEY *et al.*, 1965; SANGER *et al.*, 1965) are now well known. Regions of base pairing may also occur in HnRNA or in mRNA. Digestion with ribonucleases of the single

stranded portions of RNA would leave an intact dsRNA.

#### 1-7-4 *Self Replication of RNAs?*

Some cellular species may undergo self replication. These could be autonomous RNAs, either derived or not derived from the cell DNA. Of course the idea of independent self replication of an RNA molecule is a departure from the central dogma which states that all RNA is directly transcribed from the DNA in the normal animal cell. There is no reason that replication of a small or large part of an RNA species could not take place however. It may be the basis of some unexplained cellular function or information transfer.

#### 1-7-5 *Cell-to-Cell Communication*

A flow of genetic information from RNA to DNA was postulated in the oncogene theory (HUEBNER and TODARO, 1969). TEMIN, (1971) has further suggested in his provirus hypothesis that he envisages information from DNA in cell A being transcribed into an RNA. This RNA is then passed to cell B, where a reverse transcriptase uses the transferred RNA as a template to make new DNA. This DNA is then integrated into the DNA of cell B. The existence of the oncogenic viral-reverse transcriptase is now well documented. (SPIEGELMAN, 1970; SPIEGELMAN *et al.*, 1970; CAVALIERI and CARROL, 1970, 1971; BALTIMORE, 1970; SCOLNICK *et al.*, 1971; PENNER, COHEN and LOEB, 1970, 1971; TEITZ, 1971; CULITON, 1971) and is now extremely important in understanding carcinogenic events and the nature of cancer cells. (GALLO, YANG and TING, 1970; SCHLOM, SPIEGELMAN and MOORE, 1971; GALLO *et al.*, 1971;

nuclear RNA sequences (PHILIPSON *et al.*, 1971). The

question remains as to whether poly(A) and dsRNA are found



MULLER *et al.*, 1971; ACKERMAN *et al.*, 1971). It is interesting to speculate whether a double stranded form of RNA is the most stable for passing of information from one cell to the next. This mechanism could be involved in transfer of information in the antigenic response, between two cells.

#### 1-7-6 *Combinations of Different Types of dsRNA?*

##### *Further Queries Arising from Suspected Origins*

dsRNA isolated from cells may be a combination of all or some of these listed possibilities, each type of dsRNA having a different origin and function. It has been already reported that two different sized dsRNA molecules could be isolated (RYSKOV *et al.*, 1972).

The last two sections (1-7-3 and 1-7-4) suggest further enquiry. If dsRNA is part of another molecule, from which type of RNA (or RNA's) does it arise? What is the function of a double stranded region within a single stranded molecule? Is it a site of protection of a particularly valuable sequence of single stranded RNA, or is it the duplex form which is necessary for the attachment of a particular enzyme or protein? Does dsRNA have any effect on protein factors or polyribosome accumulation if it is involved in protein synthesis?

The coexistence of poly(A) sequences was reported by KRONENBERG and HUMPHREYS, (1972). The significance of association between polyadenylate and dsRNA was not discussed however. There is increasing evidence that post transcriptional polyadenylation is required for stabilization of nuclear mRNA sequences (PHILIPSON *et al.*, 1971). The question remains as to whether poly(A) and dsRNA are found

together in normal animal cells, e.g. rat liver, and whether or not dsRNA shares a common origin or precursor relationship with polyadenylate-associated sequences.

In section 1-7-4, the possibility of self replication is examined. If this idea of origin is entertained, what is the function of an autonomous dsRNA species capable of self replication? Is duplication of mRNA or mRNAs a real possibility? Is there any evidence for dsRNA acting as a template for further RNA synthesis? What purpose would this achieve in the overall picture of genetic inheritance?

#### 1-8 RATIONALE FOR THE WORK UNDERTAKEN IN THIS THESIS

Our present knowledge of dsRNA is particularly deficient in the areas outlined in the preceding section, and it is the purpose of this thesis to provide fundamental information to answer some of the questions given above. To achieve this purpose, it required investigation from several different approaches, with the primary aim being a thorough search into the physico-chemical properties of dsRNA and an assurance that the material prepared, not only fitted the narrowly defined criteria of a double stranded molecule, but that it was a pure preparation. As mentioned above, much of the work reported so far had been very preliminary, and concerned with the isolation of crude ribonuclease resistant fractions. In this thesis an attempt will be made to apply strictly the conditions used to define a dsRNA species, and hence to ensure the preparation of a pure dsRNA. <sup>Investigation of these details</sup>  
~~All of these aspects~~ are needed before considering questions

of cellular and molecular origin.

In the experiments in this thesis, rat tissues, particularly rat liver, were chosen as the primary source of material for the following reasons:

- (1) It afforded the opportunity to study the biosynthesis and properties of a natural molecule isolated from an "*in vivo*" situation.
- (2) Rat liver RNA properties and synthesis have been extensively studied by other workers. Methods of extraction of particular species of RNA, or in the preparation of "clean" (with low amounts of cytoplasmic contamination) nuclei or cytoplasmic fractions, had also been well defined.
- (3) Rat liver in particular is a large organ, and could yield milligram quantities of total cellular RNA, bearing in mind that dsRNA represented only a 1% fraction of this dsRNA. The disadvantages of a small quantity of dsRNA, e.g. as isolated from tissue cultured cell systems for the purpose of analysis, or to judge the effect on another system(s), are obvious.
- (4) There are some advantages of a tissue cultured system; the ease of radioactive labelling and the consequent isolation of highly radioactive species, being amongst them. However, liver cells are also metabolically active. Furthermore, a method of intravenous labelling was developed which was satisfactory and gave highly labelled species. ~~Labelling in this way could be more closely monitored, and more closely~~



~~resembled the natural "in vivo" state.~~

(5) Some preliminary studies of a crude ribonuclease resistant fraction had been undertaken (MONTAGNIER, 1968; HAREL and MONTAGNIER, 1971). It was valuable to extend this study.

(6) It offered the possibility of investigating <sup>likely</sup> ~~possible~~ interactions in which dsRNA may have been involved, e.g. protein synthesis, binding to factors, etc. These other systems had also been well defined and investigated in rat tissues.

(7) Finally the possibility of viral contamination of all rat tissues <sup>is considered</sup> ~~was~~ unlikely.

## CHAPTER II - ISOLATION AND PURIFICATION OF dsRNA FROM WHOLE CELL RNA

### 2-1 INTRODUCTION

Intact dsRNA is isolated from the cell by extracting the whole RNA from the cell and purifying and fractionating this RNA to isolate the double stranded fraction. The aim of this section is to discuss the discovery of a double stranded molecule of RNA, and to describe the isolation and fractionation procedures adopted and the particular merits of these procedures.

Methods of general RNA extraction from the whole cell are similar for all kinds of RNA from varying sources. These involve the separation of the RNA components from their interacting proteins. Generally, the preferred method is to isolate the particular cellular (or whole cell) fraction and remove the protein with phenol (or phenol-cresol or phenol-chloroform mixtures) in the presence of a detergent such as sodium dodecyl sulphate (see review of methods by STEELE and BUSCH, 1967; POULSON, 1973). This produces a large amount of crude RNA which can be further purified by removing contaminating protein with the enzyme protease, and DNA with deoxyribonuclease. The actual method adopted for preparing whole cell RNA, is taken from the techniques of AMANO (1967), and subsequently modified by NAORA and KODAIRA (1969), but it essentially consists of disruption of ~~RNA~~ whole cells by homogenization, and extraction at room temperature using sodium dodecyl

sulphate and phenol. The cellular RNA is purified by repeated ethanol precipitation. This represents intact RNA from cellular structures.

~~The principle of isolation of a double stranded RNA is then to analyse the RNA extracted from the cell as a whole and discover whether a dsRNA exists as part of the total RNA extracted. To achieve this, it is necessary to recognise several factors which distinguish dsRNA from the single stranded RNA which makes up the bulk of the extracted RNA. These particular properties of dsRNA allow the isolation and purification methods to be followed.~~

Double stranded RNA can be isolated by virtue of the fact that under certain ionic conditions it is resistant to the attack of ribonucleases. The hydrogen bonding of the double stranded molecule stabilizes the molecule and prevents the attack of the phosphodiester bond by specific ribonucleases. This specific property allows the digestion of single stranded RNA, leaving the intact double stranded RNA, provided the ionic conditions also stabilize the double stranded molecule.

In practice, therefore, the principle involves firstly, purification of the whole cell RNA by two means: (1) Treatment with protease to remove all traces of protein and to dissociate ribonuclease resistant ribonucleoprotein complexes into single stranded RNA. Such complexes, if not treated with protease, would be a source of single stranded RNA, after ribonuclease digestion, due to their resistance to



ribonuclease and consequently this would contaminate ribonuclease resistant RNA fragments remaining. (2) Treatment with deoxyribonuclease, to digest any DNA. Although the method used for general RNA extraction is designed to avoid DNA extraction, any DNA which is extracted, being also double stranded in character, would naturally render more difficult the procedures to establish the double stranded character of a ribonuclease resistant RNA. Hence the deoxyribonuclease treatment is designed specifically to remove any possible contaminating DNA.

Secondly, the specific digestion of the single stranded fragments of RNA under the optimum ionic conditions, will leave the ribonuclease resistant and probable dsRNA intact and remaining. This specific ribonuclease digestion of single stranded RNA is carried out using two enzymes, ribonuclease A and ribonuclease  $T_1$ . Ribonuclease A is derived from beef pancreas and is known because of its specific property to attack the phosphodiester linkage of the single stranded backbone of RNA involving the pyrimidine nucleotides (U and C) only. Ribonuclease  $T_1$  from a fungus *Aspergillus oryzae* is known for its specific property of cleaving the bond involving G residues. Although ribonuclease A may leave purine-rich ribonuclease resistant cores, these can successfully be degraded with ribonuclease  $T_1$ . (For details of the specific properties of these enzymes the reviews of ANFINSEN and WHITE, 1961; RAZZELL, 1967; UCHIDA and EGAMI, 1967; EGAMI and NAKAMURA, 1969,

should be consulted). Nevertheless these two enzymes specifically allow the removal of the single stranded RNA leaving intact dsRNA. When using this principle to prepare dsRNA, it is essential to investigate the ideal conditions for, (1) ribonuclease concentration. This concentration must be established to allow the digestion of all single stranded RNA ~~and not the double stranded RNA~~, and (2) achieving correct ionic environment to stabilize the hydrogen bonding of ~~a~~ <sup>the</sup> double stranded molecule.

This chapter discusses the investigation of the ionic environment, the ribonuclease:RNA ratios, and the total amount of each enzyme added. Also discussed is a comparison of the resistance <sup>of denatured and undenatured ribonuclease-resistant material to</sup> ~~to ribonuclease between any ribonuclease resistant~~ <sup>further ribonuclease treatment.</sup> ~~material which has been denatured and that which has not.~~ ~~This comparison gives an indication of the percentage of the material which is truly double stranded.~~ Investigation of all of the above mentioned conditions <sup>is</sup> ~~are~~ important to establish the optimum conditions to remove single stranded RNA and hence prepare dsRNA.

In 1971, when this investigation was begun, little information about the existence of dsRNA, or the methods of isolation, had been published. All of the early investigations had centred on the accidental discovery of a ribonuclease resistant fraction remaining after ribonuclease digestion (COLBY and DUESBERG, 1969; KIMBALL and DUESBERG, 1971; MONTAGNIER, 1968; STERN and FRIEDMAN, 1979, 1971). These crude ribonuclease fractions were thought to be dsRNA or part dsRNA. However, investigations of preparative methods, and conditions of digestion were not precisely described, and some important analyses of fractions, thought

to represent dsRNA, can be questioned because of lack of attention to ribonuclease concentration for digestion, or the ionic conditions under which digestions were carried out. For example, the studies of specific properties of the ribonuclease resistant RNA (HAREL and MONTAGNIER, 1971) could be questioned for the lack of ribonuclease  $T_1$  in the general preparation. Similarly, other workers (COLBY and DUESBERG, 1969; deMAEYER, deMAEYER-GUIGNARD, and MONTAGNIER, 1971) were interested in properties of their presumed dsRNA fraction, such as interferon induction, without having given sufficient attention to basic preparation and purification procedures. The preparation of presumptive dsRNA were often crude ribonuclease resistant fractions, and the fact that observed properties may have been due to inadequate preparation and purification only serves to emphasise the importance of establishing the optimum basic conditions to remove single stranded RNA.

Having prepared a ribonuclease resistant fraction by removal of single stranded RNA, the further purification of this fraction was undertaken. In order to purify this ribonuclease resistant RNA, chromatographic techniques of Sephadex and cellulose chromatography were studied. It is clear from the analysis of the ribonuclease resistant fractions, prior to chromatography, that they are not completely dsRNA alone. Ribonuclease digestion conditions cannot be designed <sup>precisely</sup> ~~absolutely~~ <sup>enough</sup> to prepare fractions which do not have some contamination with short nucleotide single stranded fragments



of RNA. Chromatographic methods, however, should be able to separate any two species of RNA which are dissimilar in character. Techniques of sucrose density gradient fractionation, molecular weight sieve chromatography or electrophoresis, are traditionally used to separate any two species of RNA. Consequently the separation of small RNA segments from the bulk of dsRNA in a ribonuclease resistant RNA should be achieved by chromatographic separation.

There are two aspects of the use of chromatography to be considered. The first is the use of chromatography to analyze ribonuclease resistant RNA, i.e. to discover something about its size and behaviour. The second aspect is the use of chromatography to purify the ribonuclease resistant RNA, i.e. to remove contaminating small single strands of RNA from the preparation of true dsRNA. This chapter is concerned primarily with the latter, particularly in developing an <sup>satisfactory</sup> ~~ideal~~ purification technique for dsRNA preparation. The former aspect will also be mentioned but the detailed properties of purified dsRNA are given in the following chapter. It is this consideration that is an important distinguishing difference between the work described in this chapter and the earlier work on other ribonuclease resistant fractions isolated from uninfected material (MONTAGNIER, 1968; STERN and FRIEDMAN, 1970, 1971; KIMBALL and DUESBERG, 1971). The primary concern of these workers was the use of chromatography to identify size and behaviour, rather than to explore the chromatographic method to purify their material.

Finally, one other aspect of RNA purification is investigated. The details of the separation methods used by other workers will be discussed more fully in the Discussion section of the chapter, but of the various methods which are applicable to separate different RNA molecules, two are considered in this chapter; these are Sephadex and cellulose chromatography. The Sephadex method is widely used for separating molecules on the basis of their molecular weight, *viz* it acts <sup>as</sup> ~~like~~ a molecular weight sieve. HAREL and MONTAGNIER (1972), mentioned the use of G-200 Sephadex to remove small nucleotide fragments, but gave no detail of the purification, KIMBALL and DUESBERG (1971), mentioned partial purification using 6% agarose gels. Sephadex separation (G-50 and G-200) methods are investigated for adequately removing the small single stranded RNA fragments, but limitations of the Sephadex method become obvious when further analysis of the "purified" material was undertaken. An alternative ~~unique~~ separation is investigated. The technique involves stepwise elution of the material from a cellulose column. The particular advantages of cellulose are elaborated later, but the method was originally described by FRANKLIN (1966), and its virtue appears to lie in a separation due to conformation of the molecule rather than molecular weight. It seems to be an ideal method for the purpose of separation of a purified double stranded molecule from other contaminating material. The details of separation by this method are discussed later in this chapter.

Finally, one other aspect of dsRNA purification is investigated. This involves analysis of the method used to extract RNA from the cell itself. This enquiry is the result of reported observations that differential extraction of RNA's can result from the concentration of sodium dodecyl sulphate employed and/or the temperature of the phenol extraction. Temperature and ionic strength can determine the molecular species of RNA that are extracted from cells or nuclei by phenol (SIBATANI, 1966; GEORGIEV, 1967). In the absence of detergent, for example, nuclear material which is rich in DNA-like RNA remains at the interface after phenol extraction at low temperature. Similarly this interface may contain a higher proportion of the dsRNA. Differential extraction of dsRNA may occur because of the detergent concentration, ionic conditions or phenol extraction temperature employed. Indeed MONTAGNIER (1968), reported differential extraction of dsRNA. To investigate this possibility, further extractions of the interface layer obtained after the first phenol extraction, were conducted. The results of these experiments are reported in this chapter.

In summary, this chapter aims to establish the existence of dsRNA in the rat liver cell and to investigate the methods of isolation, to prepare ribonuclease resistant RNA using the particular technique of digestion with ribonucleases A and  $T_1$  each with specific digestion properties under the appropriate ionic conditions, and to investigate



## 2-2-1 Materials

this ribonuclease resistant RNA for double stranded character. Preliminary investigations are conducted employing the specific property of susceptibility of the heat-denatured dsRNA form to further ribonuclease digestion. Secondly, the purification method is examined with the aim of preparing dsRNA and separate from any small RNA fragments which are not susceptible to ribonuclease digestion, and finally to establish whether any differential extraction of dsRNA occurs.

dsRNA was purchased from the Sigma Chem. Co. and purified by a modification of the method of FRAENKEL-CONRAT *et al.* (1961). All sucrose solutions prepared for this study were made with a special grade of ribonuclease free sucrose (Schwartz-Mann). Rats used were Wistar males (body weight 110-160 g) fed *ad libitum*, and were normally starved (unless otherwise indicated) for 24 h prior to liver removal to prevent the contamination of glycogen in the RNA preparations. Reovirus was provided by Dr. Macleod, CSIRO, Sydney, N.S.W. and dsRNA was isolated from reovirus as described by KIRBY (1963).

## 2-2-2 Radioactive Labelling

Labelling with [ $^3\text{H}$ ] orotic acid and [ $^{32}\text{P}$ ] was carried out by injection of 300  $\mu\text{Ci/head}$  of [ $^3\text{H}$ ] orotic acid or 5M Ci/head of [ $^{32}\text{P}$ ]. Injections of isotope were either intraperitoneally or directly into the hepatic portal vein after mild anaesthetization with ether.

## 2-2-3 Procedure for Administration of Isotope

This procedure was relatively simple, and involved incising the anterior abdominal wall. The incision was

## 2-2 MATERIALS AND METHODS

### 2-2-1 *Materials*

[5-<sup>3</sup>H] orotic acid (25-30 Ci/mmole) and [<sup>32</sup>P] as orthophosphate, were purchased from the Radiochem. Corp., Amersham, and the Australian Atomic Energy Commission respectively. Deoxyribonuclease (ribonuclease free) and ribonuclease T<sub>1</sub> (from *Aspergillus oryzae*) were purchased from the Worthington Biochem. Corp., pancreatic ribonuclease A and protease (from *Streptomyces griseus*) were from the Sigma Chemical Co., cellulose was Whatman CF-11 cellulose. Sephadex G-200 and G-50 were purchased from Pharmacia, Bentonite was purchased from the Sigma Chem. Co. and purified by a modification of the method of FRAENKEL-CONRAT *et al.* (1961). All sucrose solutions prepared for this study were made with a special grade of ribonuclease free sucrose (Schwarz-Mann). Rats used were Wistar males (body weight 130-160 g) fed *ad libitum*, and were normally starved (unless otherwise indicated) for 24 h prior to liver removal to prevent the contamination of glycogen in the RNA preparations. Reovirus was provided by Dr. Maclean, CSIRO, Sydney, N.S.W. and dsRNA was isolated from reovirus as described by KIRBY (1965).

### 2-2-2 *Radioactive Labelling*

Labelling with [<sup>3</sup>H] orotic acid and [<sup>32</sup>P] was carried out by injection of 300  $\mu$  Ci/head of [<sup>3</sup>H] orotic acid or 5M Ci/head of [<sup>32</sup>P]. Injections of isotope were either intraperitoneally or directly into the hepatic portal vein after mild anaesthetization with ether.

### 2-2-3 *Procedure for Administration of Isotope*

This procedure was relatively simple, and involved incising the anterior abdominal wall. The incision was

approximately 3-4 cm long. The stomach and part of the small intestine were displayed to expose the hepatic portal vein. Two silk ligatures were passed immediately beneath the vein so as to avoid tributaries from the small intestine. The portal vein was clamped with a small artery clamp as close to the liver as possible to produce dilation of the vein. The caudal ligature was tied and a 26 g needle introduced into the vein immediately cephalic to the ligature. To retain the needle in position, the other ligature was tied firmly around the needle and vein. The temporary clamp was then removed and the isotope dissolved in 0.33 ml of 0.14 M NaCl/head introduced. As the needle was withdrawn after the administration of isotope, the second ligature was tied firmly. The intestine was properly replaced and the incisions closed with Michel clips. Unless otherwise stated, rats were then sacrificed after 60 min and their livers removed.

Labelling in this way has the definite advantage of achieving a much higher concentration of isotope in the liver than could be attained by intraperitoneal injection, and hence a higher specific activity of the RNA species is obtained.

#### 2-2-4 *Isotope Solutions for Injection*

Isotope solutions for injections were prepared as follows:

[<sup>3</sup>H] orotic acid solution: 0.3 ml (300  $\mu$  Ci of [<sup>3</sup>H] orotic acid



injected/head + 0.03 ml of 1.4M NaCl (sterile solution)  
 [ $^{32}\text{P}$ ] solution: 0.5 ml of [ $^{32}\text{P}$ ] as orthophosphate/  
 injected/head obtained in dilute HCl.

This latter solution is heated in the containing vial in boiling water for 15 min and neutralized with dilute NaOH (0.1M) to pH 7.4. 0.05 ml of 1.4M NaCl is added to adjust the concentration of NaCl to 0.14M and mixed before injection.

#### 2-2-5 Preparation of Whole Cell Homogenates

Labelled livers were homogenized in 10 volumes of 0.25M sucrose solution containing 1% purified bentonite in a Waring blendor at 50V for 2 min at 0°C. Unlabelled livers were homogenized similarly, except that they were routinely first sieved (Endecott sieve No. 45) to remove any connective tissue or blood vessels.

#### 2-2-6 Extraction of Crude RNA Fraction

All glassware and pipettes used for extractions and purifications of RNA detailed throughout this thesis were routinely chromic acid washed. In some cases, apparatus was autoclaved instead to <sup>inactivate</sup> ~~remove~~ any contaminating ribonuclease.

Whole cell homogenates were first shaken with 0.1 volumes of concentrated solution containing 0.1M Tris-HCl, 10 mM  $\text{MgCl}_2$ , 1.4M NaCl pH 7.4 and 2% sodium dodecyl sulphate and shaken for 10 min at 20-25°C. This was followed by two, 10 min room temperature extractions with 80% phenol (dissolved in water) containing 0.1% of 8-hydroxyquinoline. In each case the phenol extraction was centrifuged at 14,500 rpm in the SS-34

rotor of the RC2B Sorvall centrifuge (mean g value = 25,300) and the supernatant removed by pasteur pipette. The final supernatant was precipitated with two volumes of ice-cold ethanol and stored overnight at  $-20^{\circ}\text{C}$ .

#### 2-2-7 RNA Purification

The crude RNA fraction was further purified by digestion in the buffer containing 10 mM Tris-HCl, 1 mM  $\text{MgCl}_2$ , 50 mM NaCl pH 7.4 with deoxyribonuclease (20  $\mu\text{g}/\text{ml}$  at 80  $\text{OD}_{260}$  nm/ml of RNA) and digestion subsequently with preincubated protease (100  $\mu\text{g}/\text{ml}$  at  $37^{\circ}\text{C}$  for 30 min and 60 min respectively). Protease, 1 mg/ml dissolved in the same buffer as used for digestion, was preincubated for 2 h at  $37^{\circ}\text{C}$  to remove contaminating ribonuclease in the preparation. The RNA was then re-extracted with sodium dodecyl sulphate and phenol as described above (1 extraction) and purified by precipitating RNA from the supernatant with two volumes of cold ethanol at  $-20^{\circ}\text{C}$ . This was centrifuged after a minimum of 2 h at 4,000 rpm in the RC2B Sorvall centrifuge (10 min), the precipitates redissolved in 10 mM Tris-HCl, 1 mM  $\text{MgCl}_2$ , 0.3 M NaCl pH 7.4 and re-precipitated with two volumes of cold ethanol at  $-20^{\circ}\text{C}$ . This procedure was repeated twice more. The final cellular RNA was dissolved in the requisite buffer and if necessary stored at  $-20^{\circ}\text{C}$ . In preparations where cellular RNA had been prepared from rat liver containing large amounts of glycogen, the final solution was frozen and rethawed in ice water and centrifuged at 10,000 rpm (12,100 x g) in the SS-34 rotor of the RC2B Sorvall centrifuge to remove glycogen. Glycogen was also routinely removed during chromatography.

### 2-2-8 Preparation of the Ribonuclease Resistant RNA Fraction

Ribonuclease resistant RNA was prepared from purified RNA by ribonuclease digestion of whole RNA. Digestion was carried out in 'high salt' buffer, 10 mM Tris-HCl, 1 mM  $\text{MgCl}_2$ , 0.3 M NaCl pH 7.4, using pancreatic ribonuclease A (20  $\mu\text{g/ml}$  at 80  $\text{OD}_{260}$  nm/ml of RNA) and ribonuclease  $\text{T}_1$  (1  $\mu\text{g/ml}$ ) at 37°C for 30 min. After digestion, concentrated sodium dodecyl sulphate solution (0.1 M Tris-HCl, 10 mM  $\text{MgCl}_2$ , 1.4 M NaCl pH 7.4 and 2% sodium dodecyl sulphate) was added: the amount added was equal to 10% of the total volume of the digestion solution. This was shaken for 5 min and then an equal volume of 80% phenol (dissolved in water) 0.1% 8-<sup>h</sup>hydroxyquinoline was added and two 15 min extractions at room temperature performed. The treatment of the supernatants was as described before for RNA extraction and the material purified by repeated ethanol precipitations. This represented a crude preparation of dsRNA.

### RNA 2-2-9 Chromatography of dsRNA

The crude preparation of dsRNA was applied to either Sephadex G-50 or G-200 columns, equilibrated with 20 mM Tris-HCl, 2 mM EDTA, 0.2M NaCl, pH 7.4, and 60-drop fractions collected. The preparations were applied to a (2.5 cm x 40 cm) column, water jacketed and kept at 4°C. The fractions were monitored for absorbancy at 254 nm and collected using an LKB UltroRac fraction collector also at 4°C.



Alternatively, the crude preparation was added to a cellulose column (1.5 x 20 cm on 2.5 x 30 cm) using a technique of eluting with three different buffer:ethanol concentrations, modified slightly from the original technique described by FRANKLIN (1966). The buffer, hereafter called TSE buffer, contained 5 mM Tris-HCl, 1 mM EDTA and 0.1 M NaCl pH 6.9, and the column was successively eluted with the mixture of buffer and absolute ethanol in the ratios 65:35, 85:15, and TSE buffer alone. Fractions of 3 ml or 6 ml (depending upon the size of the column) were monitored at 254 nm for absorbancy and collected using a volumetric siphon fitted to the LKB UltroRac, and retained at 4°C. Drop collection was not possible due to change in density of the buffers.

#### 2-2-10 *Analysis of Radioactive RNA fractions*

##### *Collected*

For the analysis of acid precipitable RNA in the fractions, approximately 50 µg of non-labelled rat liver RNA was added to an aliquot of the fraction obtained as a carrier. RNA was precipitated with an equal volume of ice-cold 10% trichloroacetic acid and filtered through Reeve-Angel glass fibre filters (2.4 cm). The precipitates on the filters were washed with ice-cold 5% trichloroacetic acid (three times) and ice-cold ethanol (once) and then dried. The filters were incubated with 1 ml of NCS Solubilizer (Amersham/Searle) at 37°C for several hours and 10 ml of PPO-POPOP toluene scintillation fluid added to each vial.

These were then counted in a Beckman LS-100 scintillation counter.

Alternatively, if the whole radioactive profile, including non-precipitable RNA, was desired, an aliquot of the fraction concerned was added directly to the scintillation vial, the volume adjusted to 1 ml with water if necessary, and 10 ml of Bray's scintillation cocktail, or AQUASOL (Amersham/Searle) added directly and counted.

#### 2-2-11 *Method of Analyzing Optimum Concentrations of Ribonuclease*

##### A. Optical Density Method

Unlabelled cellular RNA (1 mg) was dissolved in 10 mM Tris HCl pH 7.4 and treated with pancreatic ribonuclease A at the indicated concentration, for 30 min at 37°C. The incubation was stopped by the addition of cold perchloric acid adjusting the final concentration of perchloric acid to 2% and kept in ice for 20 min. The resulting precipitate was centrifuged at 2500 rpm in the bench centrifuge for 5 min. The optical density at 260 nm of the supernatant was then measured. The precipitate was digested with 2% perchloric acid for 20 min at 70°C and the optical density at 260 nm ascertained.

##### B. Radioactivity Method

<sup>3</sup>H-labelled cellular RNA was treated with pancreatic ribonuclease A and/or ribonuclease T<sub>1</sub> at the desired concentration for 30 min at 37°C, in either Tris-HCl buffer pH 7.6, 2 x SSC or 0.1 x SSC (pH 7.4) (1 x SSC = 0.15 M NaCl,

0.015 M Na citrate). At the end of the incubation period 300  $\mu$ g of unlabelled rat liver RNA was added as carrier and the RNA immediately precipitated by the addition of an equal volume of 10% trichloroacetic acid. The resulting precipitate was filtered, washed, dried and counted, as described in section 2-2-10 above.

#### 2-2-12 *Heat-denaturation Treatment of RNA used for Ribonuclease Concentration Experiments*

The RNA was dissolved in either 0.1 x SSC or 2 x SSC and heated in a test tube sealed with two layers of plastic film ("Glad-wrap") and secured with rubber bands, and heated to 100°C for 10 min. The tubes were then immediately plunged into ice water and allowed to cool for 5 mins.

#### 2-2-13 *Preparation of RNA from the Interface Layer Obtained after Phenol Extraction*

The interface from the first extraction with phenol (section 2-2-6) was removed using a wide-mouthed pipette and centrifuged at 15000 rpm (27000 x g) in the SS-34 rotor of RC2B Sorvall centrifuge. The phenol was removed and the necessary interface layer washed three times with a large volume of cold ethanol, centrifuging at 2500 rpm on the bench centrifuge to remove ethanol at each stage. The precipitate was finally washed with ether and dried in a stream of dry N<sub>2</sub>. It was then suspended in 15 ml of buffer containing 50 mM Tris-HCl, 25 mM KCl, 25 mM MgCl<sub>2</sub> pH 7.4. To this was added the following: 2 mg/ml protease, 5.0  $\mu$ g/ml potassium polyvinyl sulphate and 0.35% sodium dodecyl



sulphate. This mixture was then incubated for 12 h at 37°C. To this incubated mixture was added one fifth of its volume of buffer containing 1M LiCl<sub>2</sub>, 2.5% sodium dodecyl sulphate, 0.01% Na acetate, pH 5.1 and an equal volume of 80% phenol (dissolved in water). This was heated and shaken at 65°C for 10 min, then cooled, centrifuged and the aqueous layer heated as described in section 2-2-6 above.

## 2-3 RESULTS

### 2-3-1 *Preliminary Experiment to Determine Optimum Concentration of Ribonuclease A for RNA Digestion*

An attempt was made to determine the optimum concentration of ribonuclease A for complete digestion of ribonuclease A sensitive regions of RNA, ~~by the technique of measuring the optical density of nucleotides released into supernatant of ribonuclease digested RNA. The insensitive regions were precipitated with perchloric acid. These precipitates were then further measured for release of nucleotides after complete perchloric digestion.~~

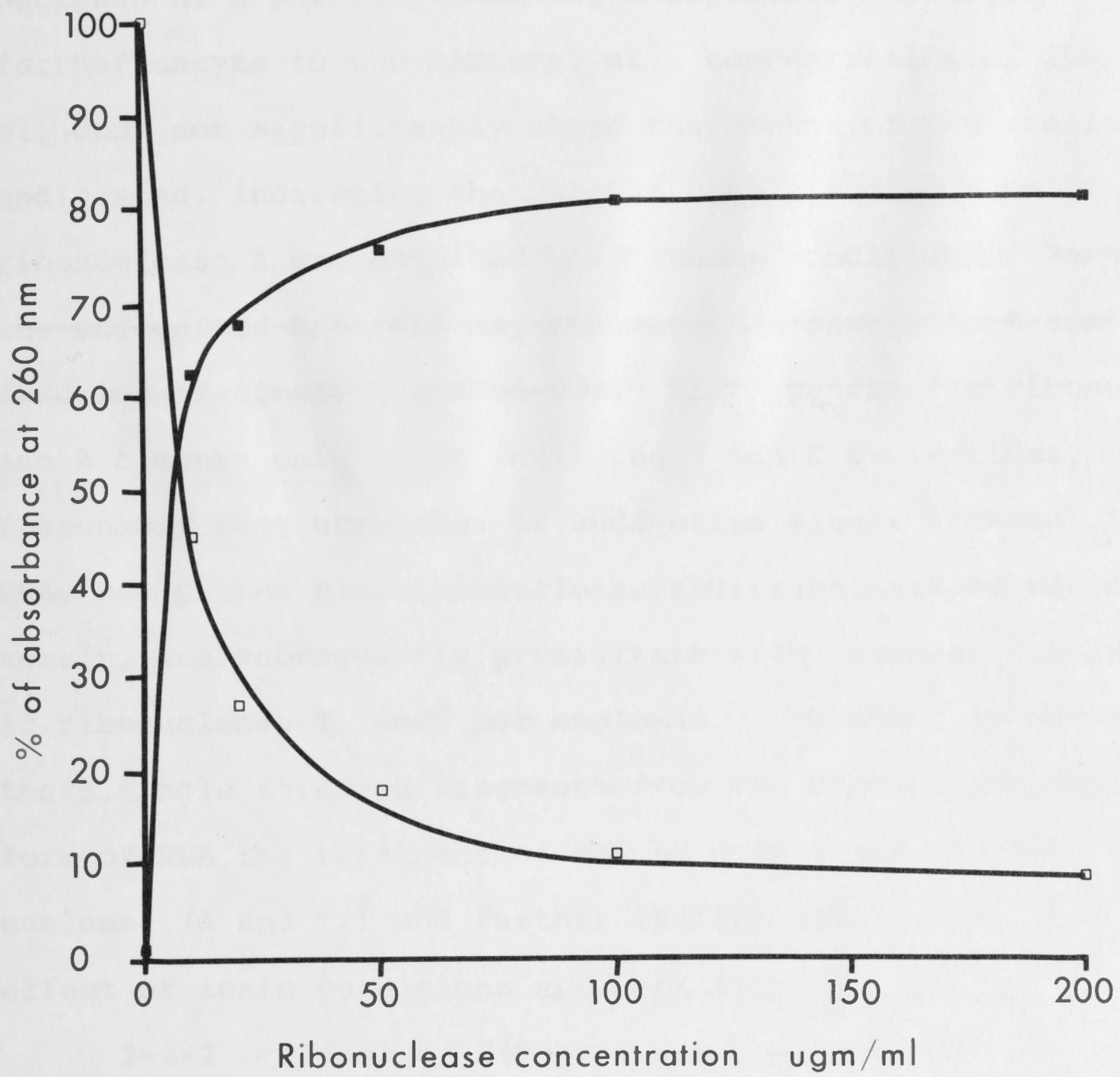
The results of this experiment are presented in Fig. 2-1. In this experiment the ribonuclease:RNA ratios varied from 1:100 to 1:5. The upper curve represents the percentage of the total optical density released into the supernatant obtained by centrifugation of a preparation of unlabelled cellular RNA digested with varying concentrations of ribonuclease A. Conversely the lower curve reflects the decrease in optical density obtained when the remaining

FIGURE 2-1     The effect of increasing ribonuclease A concentration on the yield of ribonuclease resistant RNA from cellular RNA

Whole cellular RNA isolated from rat liver (1 mg aliquots in 0.2 ml of 10 mM Tris-HCl, pH 7.4) were treated with increasing concentrations of ribonuclease A at 37°C; 30 min. Incubations were stopped by addition of 2% perchloric acid and kept on ice for 20 min. The ordinate indicates the percentage absorbance of the supernatant fraction after centrifugation, and the percentage absorbance of the subsequently digested precipitate (2% perchloric, 70°C, 20 min) of the absorbance of each whole RNA fraction.

■ ————— ■ , percentage absorbance of supernatant

□ ————— □ , percentage absorbance of digested precipitate





undigested precipitated RNA, after each particular ribonuclease digestion, is subsequently digested with perchloric acid. Even at low concentrations (10  $\mu\text{g/ml}$ ) a large proportion of the RNA had been digested. There is nevertheless even at a much higher concentration (100  $\mu\text{g/ml}$ ) of ribonuclease A, a portion remaining undigested. Addition of further enzyme to the mixture, at a concentration of 200  $\mu\text{g/ml}$ , does not significantly alter the amount of RNA remaining undigested, indicating that almost complete digestion of ribonuclease A was obtained under these conditions. ~~Part of the undigested material may reflect a ribonuclease A resistant fraction of single stranded DNA.~~ Since pancreatic ribonuclease A cleaves only bonds involving U and C nucleotides, reasonably long stretches of undigested single strands of RNA, *viz* purine rich ribonuclease resistant strands would remain, and subsequently precipitate with perchloric acid if ribonuclease  $T_1$  were not employed. In order to remove these single stranded fragments from the double stranded form of RNA the treatment of RNA by both types of ribonuclease (A and  $T_1$ ) and further characterization of the effect of ionic conditions are certainly required.

#### 2-3-2 *Effect of Ribonuclease Concentration and Ionic Concentration*

The results of several experiments are presented in Table 2-1, which indicates the effect of varying concentrations of ribonuclease A and ionic conditions. Since the amount of ribonuclease A and  $T_1$  resistant RNA is expected to be very small, radioactive RNA was used to increase the

TABLE 2-1

The effect of varying ribonuclease A concentration and ionic conditions on the yield of ribonuclease resistant RNA from  $^3\text{H}$ -labelled cellular RNA. 50  $\mu\text{g}$  of  $^3\text{H}$ -labelled RNA dissolved in 0.5 mls of indicated solvent. Ribonuclease A digestions were at 37°C for 30 min.

Ribonuclease A concentra- tion ( $\mu\text{g}/\text{ml}$ )	Solvent					
	10 mM Tris-HCl buffer, pH 7.4		2 x SSC, pH 7.4		0.1 x SSC, pH 7.4	
	cpm	% remaining	cpm	% remaining	cpm	% remaining
0	20,300	100	21,615	100	20,628	100
10	473	2.3	3,840	17	111	0.5
20	305	1.5	3,101	14	104	0.5
50	199	1.0	1,141	5.4	64	0.3
100	159	0.8	1,213	5.7	53	0.3
200	96	0.5	-	-	-	-

sensitivity of detection of resistant RNA. In this experiment the ratio of ribonuclease:RNA was varied from 1:5 to 2:1. Purified  $^3\text{H}$ -labelled cellular RNA was digested with varying concentrations of ribonuclease A and the percentage of acid-precipitable radioactive material which remains after digestion, indicated. It is apparent that the RNA is very susceptible to ribonuclease digestion even at low concentrations of ribonuclease A when the ionic strength is reduced. Even ribonuclease A alone is capable of effecting a 99.5% digestion of whole RNA in 0.1 x SSC. This low salt solution however, is not ideal for preparation of double stranded material. Indeed the double stranded structure of nucleic acids dissociates, and hence high ionic strengths are required to maintain the original double stranded state.

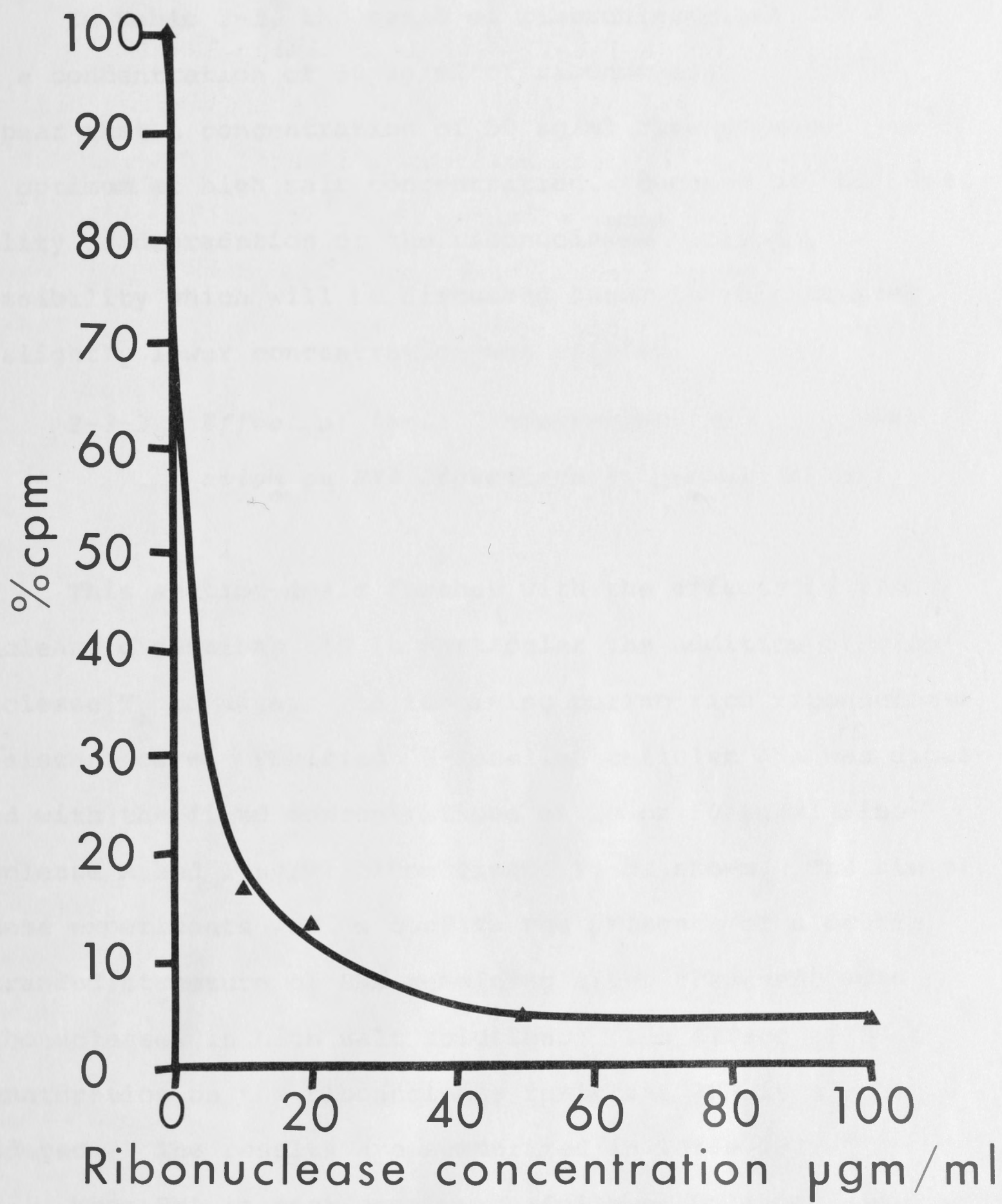
Figure 2-2 and Table 2-1, indicate the percentage of acid precipitable material remaining after ribonuclease digestion in high salt concentration, the ribonuclease:RNA ratios varying from 1:5 to 2:1. At a ribonuclease concentration of 50  $\mu\text{g/ml}$  only 5.4% of the RNA remains acid precipitable. This situation is not altered even at 100  $\mu\text{g/ml}$ , suggesting that the concentration of 50  $\mu\text{g/ml}$  is sufficient for complete digestion of ribonuclease A sensitive RNA in high salt concentration. The material which is acid precipitable is the ribonuclease resistant material which may contain dsRNA. It would also contain oligonucleotides which remain insensitive to ribonuclease A attack. It must be remembered that no ribonuclease  $\text{T}_1$  was employed and hence a



FIGURE 2-2     The effect of increasing ribonuclease A concentration on the yield of ribonuclease resistant RNA from  $^3\text{H}$ -labelled cellular RNA

Aliquots of  $^3\text{H}$ -labelled whole cellular RNA (50  $\mu\text{g}$ ,  $2.2 \times 10^4$  cpm dissolved in 0.5 mls of 2 x SSC) were treated at the ribonuclease concentrations indicated, 37°C, 30 min. The ordinate indicates percentage radioactivity remaining after digestion of the radioactivity of each whole RNA aliquot (data from Table 2-1).

▲————▲ , percentage radioactivity



purine rich ribonuclease resistant core may remain. The addition of ribonuclease  $T_1$  would digest purine rich fragments and that effect will be described in the next section.

In Table 2-1, the ratio of ribonuclease:RNA was 1:1 at a concentration of 50  $\mu\text{g/ml}$  of ribonuclease. It would appear that a concentration of 50  $\mu\text{g/ml}$  ribonuclease A would be optimum at high salt concentration. Because of the possibility of degradation of the ribonucle<sup>otide</sup>~~ase~~ backbone, a possibility which will be discussed later in this chapter, a slightly lower concentration was adopted.

### 2-3-3 *Effect of ionic Concentration and Heat Denaturation on RNA Digestions by Ribonucleases A and $T_1$*

This section deals further with the effects of ribonuclease digestions and in particular the addition of ribonuclease  $T_1$  to digest the remaining purine-rich ribonuclease resistant core. Purified  $^3\text{H}$ -labelled cellular RNA was digested with the fixed concentrations of 20 or 50  $\mu\text{g/ml}$  ribonuclease A and 1  $\mu\text{g/ml}$  ribonuclease  $T_1$  as shown. The aim of these experiments was to confirm the presence of a double stranded structure of RNA remaining after treatment with ribonucleases in high salt solution. The effect of heat denaturation on the ribonuclease resistant RNA is also considered. The results are summarized in Table 2-2.

When RNA is heat denatured, followed by immediate chilling in ice, any double stranded forms would be separated into their respective strands, the rapid cooling preventing



TABLE 2-2

The effect of ionic concentration and heat-denaturation on RNA digestions by ribonucleases A and  $T_1$ . 50  $\mu$ g of  $^3$ H-labelled cellular RNA, dissolved in 0.5 mls of the indicated solvent was either heat-denatured, ribonuclease treated or both, as indicated.

	I		II		III			
	Heat-denaturation and ribonuclease treatment in 10 mM Tris-HCl, pH 7.4		Heat-denaturation and ribonuclease treatment in 2 x SSC pH 7.4		Heat-denaturation in 0.1 x SSC, pH 7.4, and ribonuclease digestion in 2 x SSC, pH 7.4			
	cpm	% remaining	cpm	% remaining	Experiment 1		Experiment 2	
					cpm	% remaining	cpm	% remaining
1. No ribonuclease	22,624	100	22,083	100	29,150	100	23,703	100
2. 20 $\mu$ g/ml ribonuclease A for 30 min, 37°C.	134	0.6	3,179	14	4,349	14.9	3,979	16.7
3. 20 $\mu$ g ribonuclease A + 1 $\mu$ g/ml ribonuclease $T_1$ for 30 min, 37°C.	11	0.04	1,738	7.8	2,019	6.9	1,891	7.9
4. Heat-denaturation only	20,986	93	21,939	99	25,535	87.5	23,223	97
5. Heat-denaturation + 20 $\mu$ g/ml ribonuclease A for 30 min, 37°C	150	0.7	2,750	12.5	1,838	6.3	2,230	9.4
6. Heat-denaturation + 20 $\mu$ g/ml ribonuclease A + 1 $\mu$ g/ml ribonuclease $T_1$ for 30 min, 37°C	14	0.04	1,264	5.7	1,032	3.5	1,366	5.7

the reassociation of the two strands. Hence a difference in ribonuclease sensitivity before and after denaturation should give some measure of the likely percentage of double stranded material. Columns I and II of Table 2-2 illustrate the results obtained when the whole treatment, *viz* both denaturation and subsequent digestion, are carried out in low I or high II salt solution. In experiment III, the denaturation is carried out in low salt buffer to ensure separation of the strands and the degradation is then conducted after adjustment of the rapidly cooled RNA to higher salt concentrations. In addition an extra precaution was taken by retreating the whole labelled cellular RNA with deoxyribonuclease to ensure that there could be no contaminating DNA. In practice, however, this second digestion did not alter the percentage of material remaining after digestion, <sup>as</sup> and the material was totally digested by 0.3M KOH, and was thereafter omitted.

Predictably, at low concentrations of salt (column I), the ribonucleases completely degrade the RNA, leaving almost no acid precipitable RNA. The results of experiments II and III nevertheless indicate that approximately 92% digestion of the <sup>3</sup>H-labelled cellular RNA is achieved at the higher salt concentration using ribonuclease A and T<sub>1</sub> at concentrations of 20 µg/ml and 1 µg/ml respectively. At higher salt concentrations, 14% of labelled RNA remains undigested using ribonuclease A, leaving a purine rich ribonuclease resistant core. This percentage can be reduced

to 7.8% if ribonuclease  $T_1$ , specific for the cleavage of bonds involving G residues, is used to digest this core. ~~Since the double stranded form of RNA can only be maintained in high salt solution, the fraction of RNA remaining seems certain to contain dsRNA. As will be mentioned later, this fraction contains not only double stranded RNA but contaminants such as poly(A) segments (see Chapter VI).~~

The concentration of ribonuclease A used in experiments II and III, is 40% of the concentration previously observed to be desirable for complete digestion of single stranded RNA detailed in the previous section, *viz* 50  $\mu\text{g/ml}$  ribonuclease A. At this ribonuclease:RNA ratio of 1:1, degradation of dsRNA may occur. Indeed such a condition is described for the conditions of dsRNA partial digestion (LOVING and SZEKELY, 1973). To avoid causing nicks in the backbone chain of RNA, or any degradation of the dsRNA itself, it was decided to adopt the lower concentration of ribonuclease A, *viz* 20  $\mu\text{g/ml}$ , with the addition of 1  $\mu\text{g/ml}$  of ribonuclease  $T_1$ , as the optimum for preparation of intact dsRNA. Any remaining small single stranded oligonucleotides would be removed in subsequent chromatographic separation.

Treatment of the labelled cellular RNA with heat alone, caused a small degradation of the material. The percentage indicated in these experiments varies between 1 and 12%. This degradation does vary from preparation to preparation, and could be due to artificial breaks in the backbone chain in the double stranded form of the RNA and/or very small



contamination of ribonuclease itself. Even the very smallest amount of ribonuclease contamination may cause some degradation.

Almost complete digestion of RNA was observed by treatment with ribonucleases A and  $T_1$  if the treatment was carried out in the 10 mM Tris-HCl buffer alone, and hence no effect of heat denaturation was observed. ~~In contrast, the treatment of RNA by both ribonucleases in the high salt solution certainly caused a selective digestion of single stranded RNA, leaving the double stranded form of RNA remaining. This was shown in experiments II and III.~~ In experiment III, 2019 cpm remained after digestion with ribonuclease A (20  $\mu$ g/ml) and  $T_1$  (1  $\mu$ g/ml). If this same quantity of RNA is first heated in low salt, subsequent ribonuclease digestion results<sup>ed</sup> in 1032 acid precipitable cpm. This indicates not only a loss of approximately 50% in cpm due to denaturation of the likely double stranded forms, but that this form is approximately 3% of the total cellular RNA. This experiment was repeated and a value of approximately 2% was obtained under the same conditions. A similar result can be obtained with RNA denatured in high salt solution (2 x SSC, column II). Here also the apparent duplex form would appear to be 2% of the total. It should be mentioned here, that in these experiments some radioactivity remained even after heat denaturation and ribonuclease treatment. Although this may be partly due to the rapid reassociation of the RNA chains, an effect which will be described later in Chapter V, the reason for this is not clear at present.

2-3-4    *Investigation of Differential Extraction of  
the Whole Cell RNA*

An attempt was made to study whether the phenol-sodium dodecyl sulphate extraction technique differentially extracts an RNA fraction which is rich in ribonuclease resistant properties. Extracting whole liver at room temperature with sodium dodecyl sulphate and phenol leaves an interface layer after the first centrifugation. This interface layer has been the subject of investigation, and has been shown to contain RNA which contains a greater proportion of ribonuclease resistant structures than that obtained in the first phenol extraction of whole cells. This differential extraction is also dependent upon the sodium dodecyl sulphate concentration, ionic conditions, temperature, length of extraction time, etc. MONTAGNIER (1968) suggested that a fraction of RNA which was rich in dsRNA could be subsequently extracted from this interface layer. A method was developed for complete extraction of RNA from this interface layer.

The method is described under section 2-2-13, but essentially it consists of a vigorous protease digestion of the layer and subsequent phenol extraction and purification. The resulting interface layer was digested with deoxyribonuclease and ribonucleases in parallel with the digestions and purifications for the aqueous layer obtained from the same first phenol extraction.

The results are expressed in Table 2-3, which illustrates the quantity of RNA as determined by absorbance at 260 nm, the

TABLE 2-3

Comparison of the specific activities and quantities of purified RNA and ribonuclease resistant RNA fractions obtained from the aqueous and interface layers after the first phenol extraction of  $^3\text{H}$ -labelled rat liver. Methods of RNA preparation and ribonuclease digestion are described in detail in the METHODS section.

Purified RNA undigested with Ribonuclease		Isolated from Aqueous layer	Isolated from Interface layer
	Total Quantity RNA (mg)	19.6	0.54
	Total cpm	$4.01 \times 10^6$	$9.4 \times 10^4$
	Specific Activity (cpm/ $\mu\text{g}$ )	205	174
Ribonuclease Resistant RNA obtained by digestion of Purified RNA with ribonucleases A and T <sub>1</sub>	Total Quantity RNA (mg)	12.6	0.1
	Total cpm	$4.95 \times 10^5$	$6 \times 10^3$
	Specific Activity (cpm/ $\mu\text{g}$ )	39.1 (162)	58 (159)
	Percentage of undigested Purified RNA fraction	12	11



total radioactivity, and the specific activity of the purified RNA obtained from aqueous layer and interface preparations. Also indicated is the value of total radioactivity obtained in the purified ribonuclease resistant fractions as a percentage of the whole cell RNA radioactivity.

Within the limits of experimental error, these percentages indicate that there is no difference in the <sup>proportion</sup> ~~amount~~ of ribonuclease resistant RNA in the two fractions. The specific activities of the two ribonuclease resistant fractions are not <sup>noticeably</sup> ~~significantly~~ different; 58 cpm/ $\mu$ g and 39:1 cpm/ $\mu$ g. In a repeat experiment, values of 162 cpm/ $\mu$ g and 159 cpm/ $\mu$ g were obtained for aqueous and interface RNA preparations respectively.

The interface ribonuclease resistant fraction was also chromatographed on G-200 Sephadex (and the profile is shown in Figure 2-3b). No major differences in the profile for aqueous and interface fractions <sup>were</sup> ~~are~~ obvious and similar heterogeneous patterns <sup>were</sup> ~~are~~ obtained.

The percentage of the ribonuclease resistant RNA's of the undigested RNA fractions are also similar, e.g. the ribonuclease resistant fraction is 12% of the aqueous layer RNA and 11% of the interface layer. In addition, the total amount of radioactivity  $6 \times 10^3$  cpm of ribonuclease resistant RNA obtained from the interface layer is very small compared to the  $4.95 \times 10^5$  cpm obtained in the aqueous layer.

From the results outlined above, it can be concluded that no selective fractionation of the ribonuclease resistant RNA appeared to take place during the sodium dodecyl sulphate-

-phenol extraction of whole cells. For this reason and because of the poor yield of RNA from the interface layer, routine extractions of this layer were not continued.

2-3-5 *Purification of the Ribonuclease Resistant Fraction: Chromatographic Characterization of Ribonuclease Resistant RNA's*

The previous experiments established the existence of a section of RNA having double stranded character within the ribonuclease resistant RNA. The following sections deal with the characterization of the ribonuclease resistant material, with the aim of separating remaining small single stranded nucleotides from the preparation which had not been removed by phenol extractions or subsequent ethanol precipitations.

2-3-6 *Sephadex G-200 Separation*

Figure 2-3-a illustrates the profile of  $^3\text{H}$ -labelled ribonuclease resistant RNA prepared from whole cell RNA and eluted from a G-200 Sephadex column. At least two peaks are clearly distinguishable. The majority of the ultraviolet absorbing material which was <sup>partly</sup> separated from the first peak was eluted in the second peak. This material has a high absorption at 254 nm, but was not acid precipitable. This fraction represented a large proportion of short length nucleotides, not removed completely during phenol extraction. The first peak consisting of fractions 22 to 42 was rather broad, indicating that the material was heterogeneous in size.

FIGURE 2-3a and b     Sephadex G-200 Chromatography of  $^3\text{H}$ -  
labelled ribonuclease resistant RNA  
isolated from whole rat liver cells

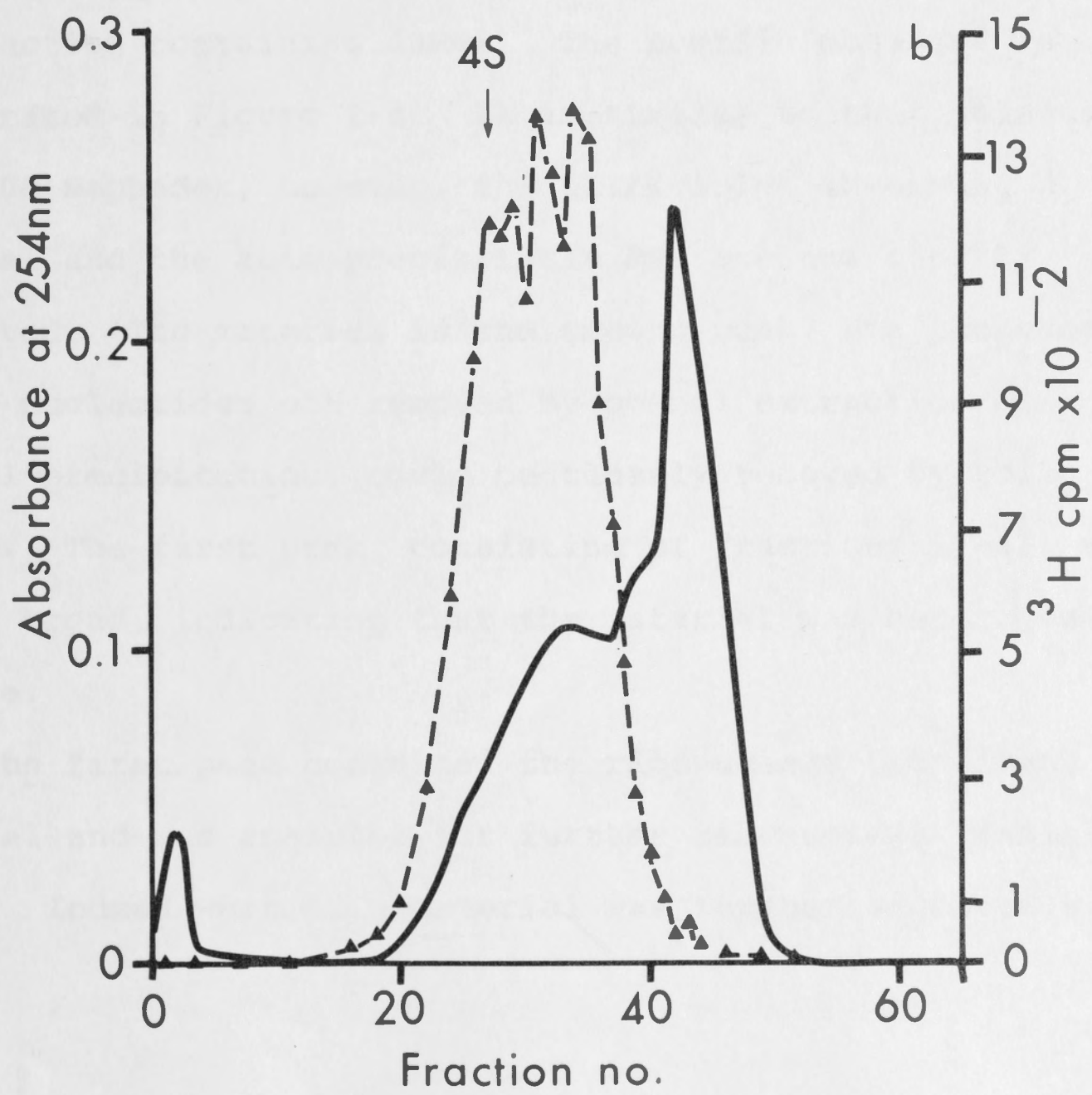
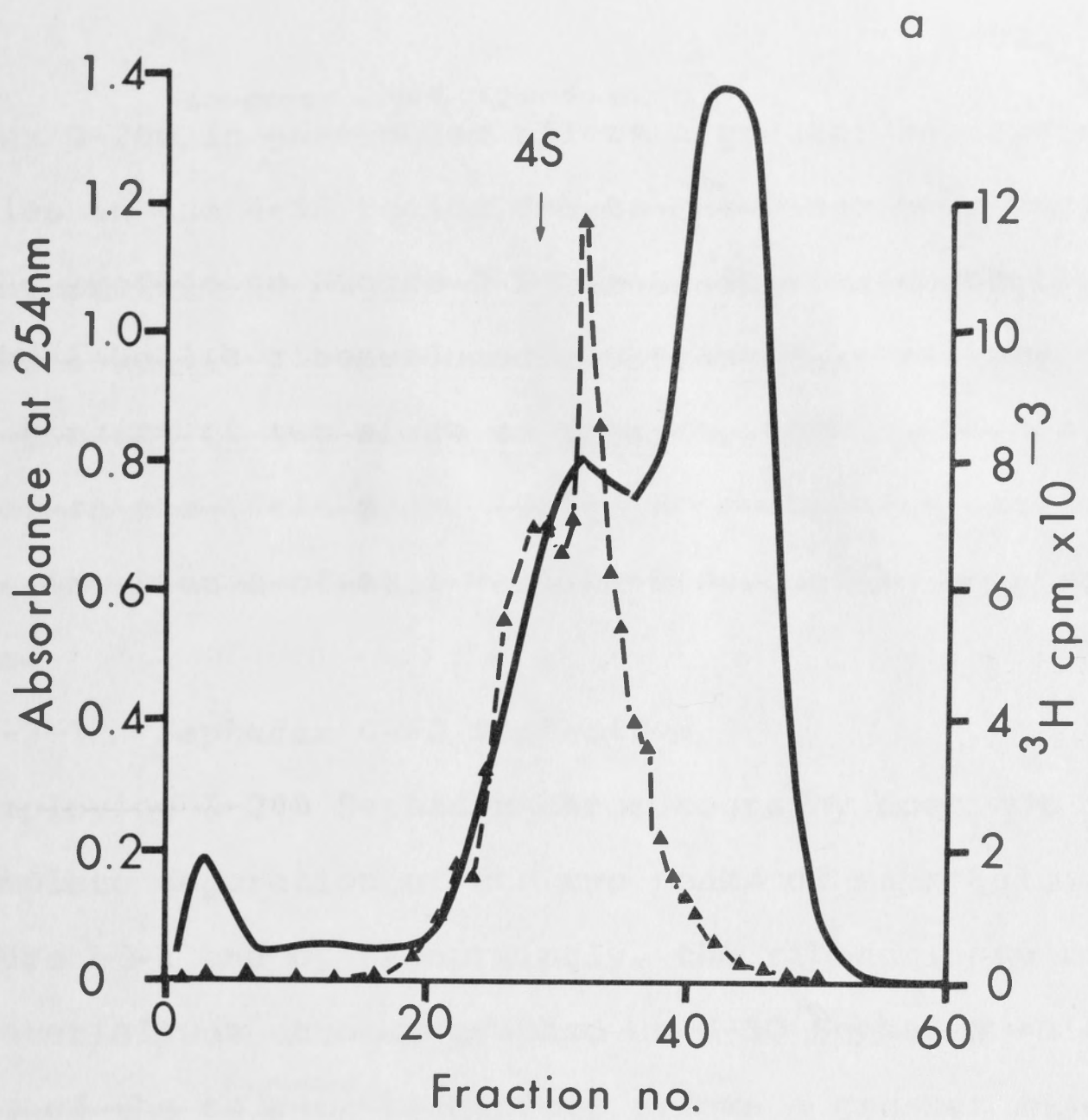
Animals had been injected intraperitoneally with [ $^3\text{H}$ ] orotic acid and sacrificed after 4 h, and the extracted RNA dissolved in buffer containing 20 mM Tris-HCl, 2 mM EDTA, 0.2 M NaCl, pH 7.4, and applied to a column equilibrated with the same buffer. Sixty drop fractions were collected and 0.5 ml aliquots were assayed for radioactivity by trichloroacetic acid precipitation, as described. The position of an intact rat liver tRNA marker (4S) is indicated.

(a) represents the  $^3\text{H}$ -labelled ribonuclease resistant RNA fraction obtained from the aqueous, and (b) the interface layer, after the first phenol extraction of the liver homogenate.

\_\_\_\_\_ , Absorbance at 254 nm

▲.....▲ , Trichloroacetic acid precipitable  
radioactivity





comparison with G-50 or G-100,  
 Sephadex G-200, in particular allows a greater separation of  
 molecules in the 4-5S region, ~~due to the finer bead size.~~  
~~From the profile in Figure 2-3-a there is some suggestion~~  
~~that the labelled ribonuclease resistant material may in~~  
~~itself consist of two sizes as there appears to be a slight~~  
~~shoulder on the first peak. This observation is noted here,~~  
~~but the importance of this will be discussed in a later~~  
~~chapter.~~

#### 2-3-7 Sephadex G-50 Separation

Employing G-200 Sephadex chromatography does not allow the complete separation of the two peaks of material indicated in Figure 2-3-a and b. Accordingly, the ribonuclease resistant material was chromatographed on G-50 Sephadex which, ~~because of the coarser bead size,~~ allows a greater separation of the very small, non precipitable nucleotides and the fraction containing dsRNA. The profile obtained is illustrated in Figure 2-4. It is similar to that obtained on G-200 Sephadex; however, the ultraviolet absorbing material and the acid precipitable RNA are now clearly separated. The material in the second peak, viz the shorter length nucleotides not removed by phenol extraction or ethanol precipitation, could be clearly removed by this method. The first peak, consisting of fractions 17-41, was rather broad, indicating that the material was heterogeneous in size.

The first peak contained the ribonuclease resistant material and was analyzed for further ribonuclease resistance. Indeed when this material was further digested with

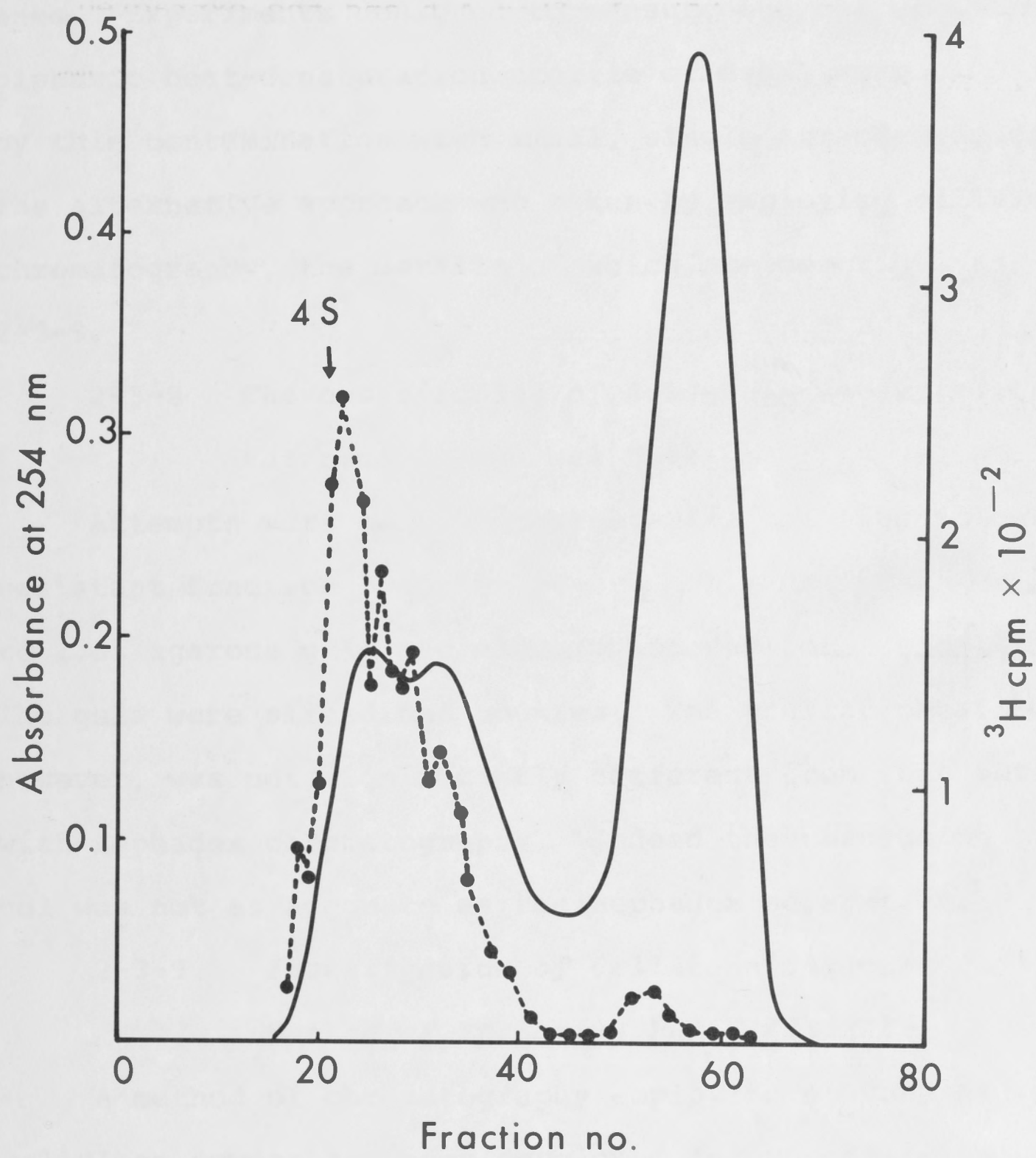
FIGURE 2-4      Sephadex G-50 Chromatography of  $^3\text{H}$ -labelled  
ribonuclease resistant RNA isolated from  
whole rat liver cells

Animals were injected intraperitoneally with [ $^3\text{H}$ ] orotic acid and sacrificed after 4 h, and the extracted RNA dissolved in buffer containing 20 mM Tris-HCl, 2 mM EDTA, 0.2M NaCl, pH 7.4 and applied to a column equilibrated in the same buffer. Sixty drop fractions were collected and 0.5 ml aliquots were assayed for radioactivity by trichloroacetic acid precipitation, as described. The position of an intact rat liver tRNA marker (4S) is indicated.

\_\_\_\_\_ , Absorbance

●.....● , Trichloroacetic acid precipitable  
radioactivity





ribonucleases after pooling and concentration, ribonuclease resistant material was certainly found in this fraction.

Moreover, this material was completely digested by 0.3N

KOH, indicating that this ribonuclease resistant fraction was only RNA. However, <sup>removal of all single stranded contaminants from</sup> ~~the G-50 Sephadex separation does~~

<sup>dsRNA may not have been accomplished.</sup> ~~not appear to completely remove all single stranded contamin-~~

~~ants.~~ Experiments designed to measure the characteristic biphasic heat-denaturation profile of dsRNA were hindered by this contamination with small, single stranded fragments. The alternative approach was taken by employing cellulose chromatography, the details of which are described in 2-3-9.

#### 2-3-8 *Characterization of Ribonuclease Resistant Material on Agarose Gels*

Attempts were made to characterise the ribonuclease resistant fraction by other means. This material was applied to 1.5% agarose gels and electrophoresed for 1 h at 10 mA/gel. The gels were sliced and counted. The profile obtained, however, was not significantly different from that obtained with Sephadex chromatography. Indeed the resolution of the gel was not as adequate as the Sephadex separation.

#### 2-3-9 *Investigation of Cellulose Chromatography Method of Preparing Purified dsRNA*

A method of chromatography employing columns of cellulose powder has been developed for successfully separating single stranded virus RNA material from double stranded viral replicative intermediate (RI) (FRANKLIN, 1966). This method was explored for the separation of

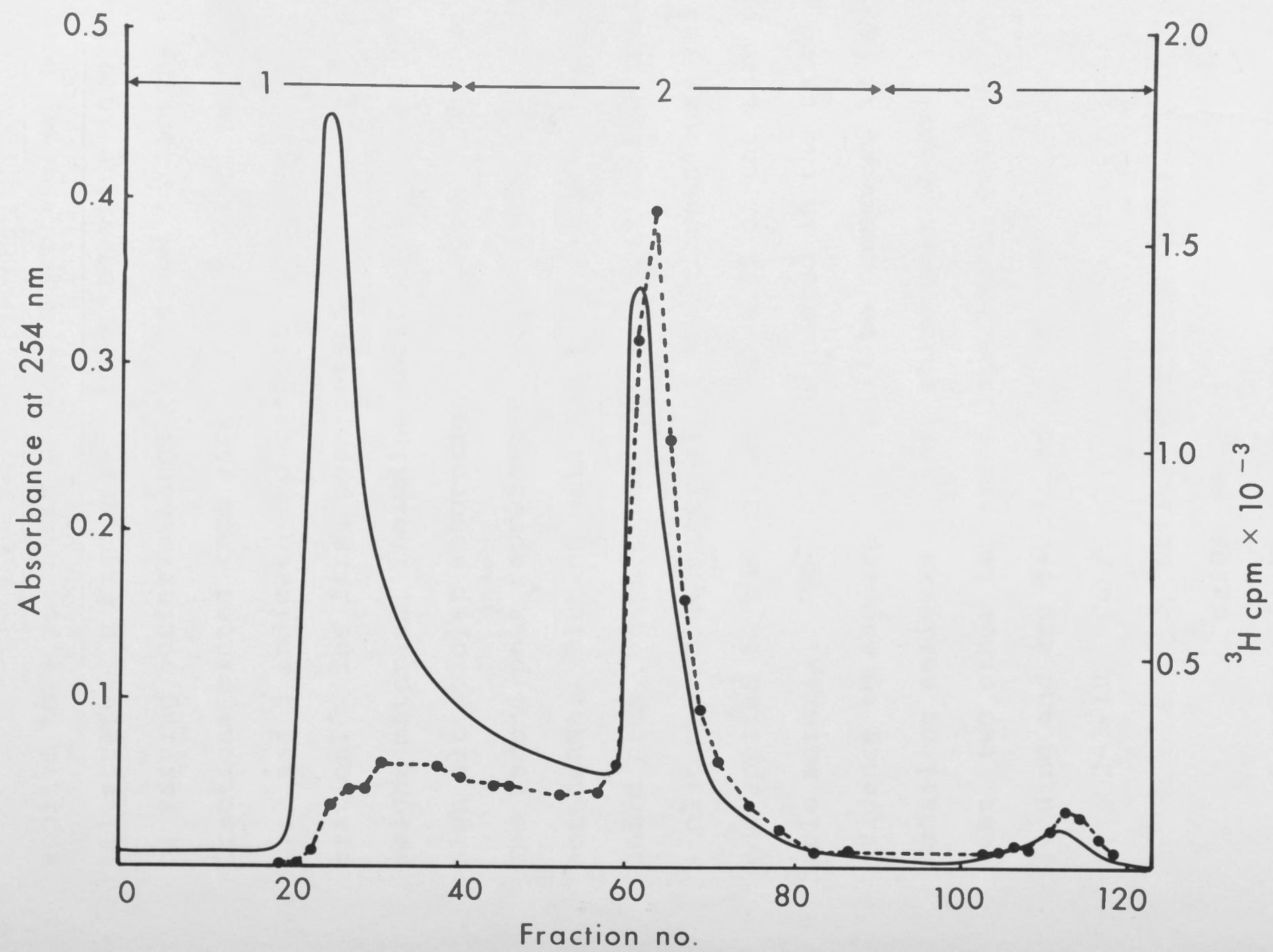
FIGURE 2-5    Cellulose column chromatography of  $^3\text{H}$ -  
labelled ribonuclease resistant RNA  
isolated from whole rat liver cells

Animals had been injected intravenously with [ $^3\text{H}$ ] orotic acid and sacrificed after 1 h. The purified ribonuclease resistant RNA was dissolved in the first TSE:ethanol solution. 1, 2 and 3 represent elution with TSE:ethanol ratios of 65:35, 85:15 and buffer alone, respectively. The RNA was applied to a 2.5 x 35 cm CF-11 cellulose column and 6 ml fractions were collected.

●.....● , Trichloroacetic acid  
precipitable radioactivity  
in 0.5 ml of each column  
fraction.

\_\_\_\_\_ , Absorbance at 254 nm.





pure dsRNA from contaminating RNA fractions in the material isolated by ribonuclease digestion.

When the  $^3\text{H}$ -labelled ribonuclease resistant RNA was applied to a cellulose column, a profile was obtained as illustrated in Figure 2-5. The technique involves the use of varying buffer:ethanol ratios and collecting the various fractions eluted from each buffer:ethanol concentration. 1, 2 and 3 indicate the respective buffer:ethanol concentration. The first peak obtained<sup>probably</sup> represented small molecular weight material, including short nucleotides, which had a high ultraviolet absorption, but low acid precipitable cpm. The second peak<sup>probably</sup> represented longer nucleotide lengths, but not dsRNA. Eluting with TSE buffer alone produced the third peak, which contained only 3.1% of the total ultraviolet absorbing material of the ribonuclease resistant RNA applied to the column, but 6.0% of the acid precipitable material. The dsRNA was eluted in the third peak, and evidence to support this will be presented in Chapter III detailing analysis of this third peak material. Nevertheless, two other important experiments substantiate this finding and are described in the next section.

2-3-10     *Confirmation that the Third Peak obtained  
by Cellulose Chromatography is Certainly  
dsRNA*

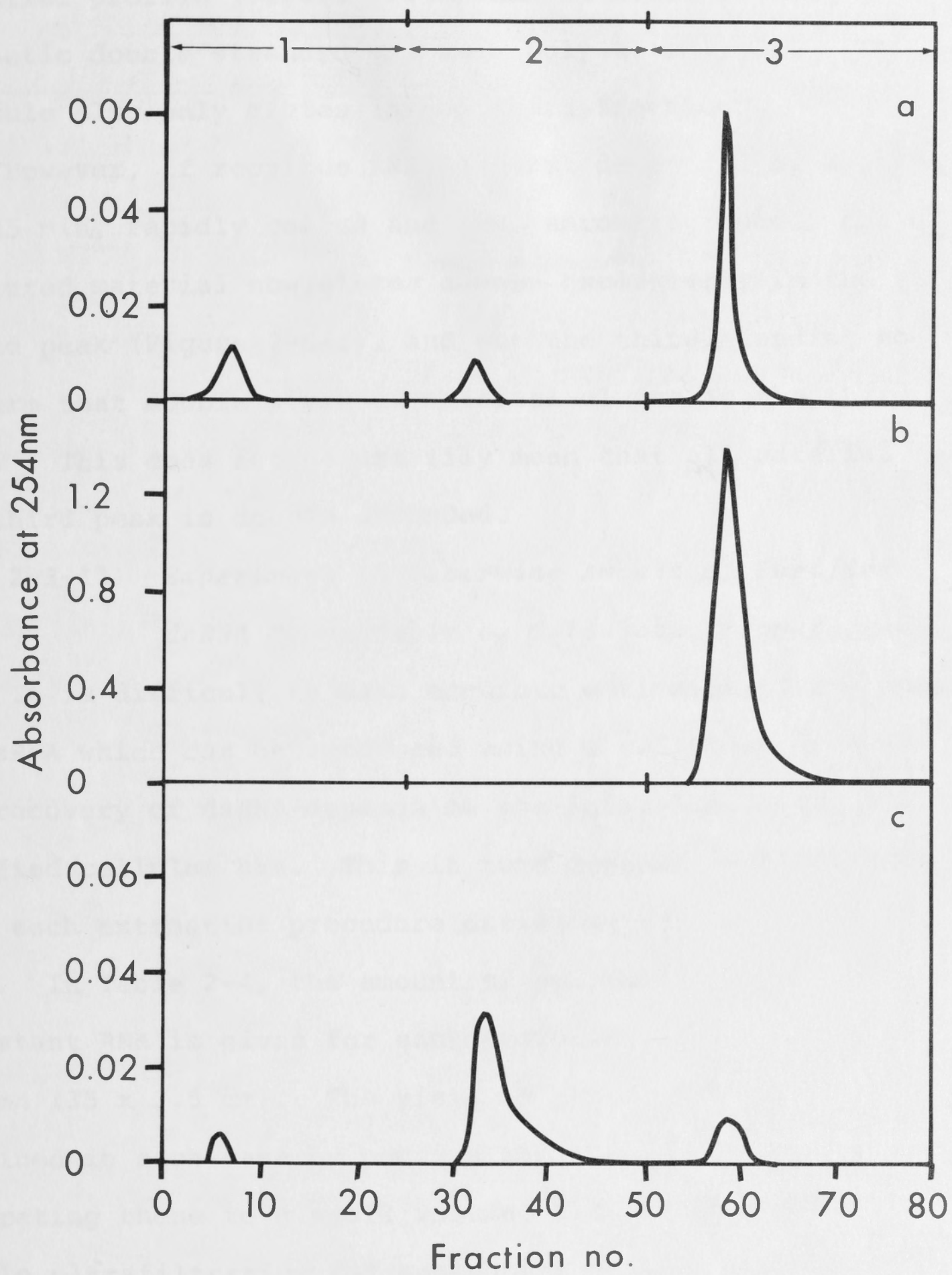
Reovirus is known to contain a naturally occurring dsRNA in the normal genetic form. Extracted reovirus dsRNA was chromatographed on cellulose in the same manner as

FIGURE 2-6-a, b and c    Cellulose column chromatography  
of preparations of (a) Reovirus  
dsRNA, (b) poly(I).poly(C) and  
(c) heat denatured reovirus dsRNA

RNA preparations were dissolved in the first TSE:ethanol solution. 1, 2 and 3 represent elution with TSE:ethanol in the ratios 65:35, 85:15 and buffer alone, respectively. Reovirus dsRNA was denatured by boiling for 10 min in 0.1 x SSC and cooling immediately in ice. The RNA was applied to 1.5 x 20 cm cellulose columns and 3 ml fractions collected.

\_\_\_\_\_, Absorbance at 254 nm.





employed for ribonuclease resistant RNA. The elution profile is illustrated in Figure 2-6-a. The third peak only is <sup>significant</sup> ~~apparent~~ and this represents the <sup>majority</sup> ~~whole~~ of the reovirus dsRNA. A similar profile (Figure 2-6-b) can be obtained with a synthetic double stranded molecule poly(I).poly(C). This molecule also only elutes in the third fraction.

However, if reovirus RNA is first denatured by heating for 15 min, rapidly cooled and then chromatographed, the denatured material now elutes <sup>most significantly</sup> ~~almost exclusively~~ in the second peak (Figure 2-6-c), and not the third, tending to confirm that double stranded material elutes in the third peak. This does not necessarily mean that all material in the third peak is double stranded.

#### 2-3-11 *Experiment to Determine Amount of Purified dsRNA Recoverable by Cellulose Chromatography*

It is difficult to make accurate estimates of the amount of dsRNA which can be recovered using a cellulose column. The recovery of dsRNA depends on the initial recovery of purified cellular RNA. This in turn depends on how quantitative each extraction procedure during purification can be made. In Table 2-4, the amount of purified ribonuclease resistant RNA is given for each individual application to the column (35 x 2.5 cm). The yield of third peak material obtained in each case by pooling the relevant fractions, concentrating these to a small volume, 2-3 ml using AMICON diaflo ultrafiltration and subsequent ethanol precipitation, is also given.

TABLE 2-4

The yield of third peak material (RNA) after cellulose chromatography of ribonuclease resistant RNA. Quantities of RNA were determined by the absorbance at 260 nm. Cellulose chromatography was conducted on a large column of cellulose (2.5 x 35 cm) as described in 2-2-9.

Expt. No.	Ribonuclease Resistant RNA applied (mg)	Yield of third peak material after con- centration and ethanol precipitation ( $\mu$ g)	Percentage yield of applied RNA
1	50	450	0.9
2	50	450	0.9
3	24	240	1.0
4	29.8	310	1.0
5	28.6	170	0.6

#### 2-4-1. Ribonuclease Digestion: Optimum Conditions

It is known that pancreatic ribonuclease A and ribonuclease T<sub>1</sub> isolated from *Aspergillus oryzae* do not attack a double stranded chain of RNA when the ribonuclease:RNA ratios are low and the optimum conditions for the stability of the double stranded molecule are present. This property can be used to prepare dsRNA from the bulk of single stranded RNA extractable from the cell. Ribonuclease



In each case the value of actual recovery is given as a percentage of the amount applied. These values are all based on optical density (260 nm) measurements. The recoveries vary very little and the method is quite reproducible. This applies even to ribonuclease resistant RNA amounts as high as 50 mg applied to the column.

## 2-4 DISCUSSION

This chapter has dealt with several important aspects of dsRNA. Firstly, the existence of a ribonuclease resistant in rat liver purified cellular RNA is confirmed. From the behaviour of this ribonuclease resistant RNA on Sephadex chromatography, it appears that this ribonuclease resistant fraction is similar to that described by MONTAGNIER (1968). There are, however, several differences in the preparation procedures adopted.

The procedures adopted for the preparation of ribonuclease resistant RNA have been ascertained by careful consideration of the dsRNA form, and its properties. In particular, a detailed study of the optimum ribonuclease conditions and the ionic concentrations for digestion, have been attempted.

### 2-4-1 Ribonuclease Digestion: Optimum Conditions

It is known that pancreatic ribonuclease A and ribonuclease  $T_1$  isolated from *Aspergillus oryzae* do not attack a double stranded chain of RNA when the ribonuclease:RNA ratios are low and the optimum conditions for the stability of the double stranded molecule are present. This property can be used to prepare dsRNA from the bulk of single stranded RNA extractable from the cell. Ribonuclease

digestion of the single stranded component is the preferred method of preparation of the dsRNA, for two reasons. If the dsRNA occurs as part of another larger single stranded molecule, then the ribonuclease will remove the larger chain portions - leaving only the dsRNA fragment intact. A second practical reason for choosing ribonuclease digestive methods involves the quantities of RNA extracted. Other properties of dsRNA that differ from those of single stranded RNA, and which could be applied for separation, e.g. differential solubility, are not normally employed for large scale preparation of ribonuclease resistant material. The small quantity of dsRNA capable of isolation (approximately 1%) cannot practically be isolated by any other method than first isolating a ribonuclease resistant fraction and subsequently purifying this fraction by isolation of the truly double stranded form. In choosing the digestive method, one can also satisfy one of the first requirements for dsRNA, i.e. that it be ribonuclease resistant. This method must be carefully controlled to fulfill the requirements for adequate preparation.

The theory behind the use of ribonuclease to prepare dsRNA, lies not only with the properties of dsRNA, but with the properties of the enzymes ribonuclease A and  $T_1$  themselves. Indeed dsRNA can be prepared because a large bulk of single stranded RNA is susceptible to these enzymes in two ways. Pancreatic ribonuclease A attacks the phosphodiester linkage of the single stranded backbone of RNA involving the pyrimidine nucleotides (U and C) only.

have the disadvantage of causing nicks in the phosphodiester

Ribonuclease  $T_1$  cleaves the bonds involving G residues (EGAMI and NAKAMURA, 1969). The combined action of these enzymes therefore, provides a method for satisfactory removal of single stranded RNA. However, this digestive method can only operate if the ionic conditions are such that they stabilize the hydrogen bonding of the double stranded molecule. It can be seen clearly from the experiments in this chapter that the ribonuclease resistant-presumptive dsRNA regions can only exist if there is sufficient ionic strength in the form of sodium chloride concentration to maintain those regions. If the ionic strength is low, the double chain presumably dissociates into separate strands and is digested by ribonucleases. Therefore high salt concentration is mandatory for the preparation of intact dsRNA.

Not only is a high ionic strength required to prepare intact dsRNA, but careful attention must be paid to the actual concentration of ribonucleases employed to remove single stranded RNA. It is important to employ digestive conditions which exclude the possibility of double stranded degradation. Employing high ionic strength does not necessarily mean there is no double stranded degradation. The resistance of dsRNA is only relative. BISHOP and LEVINTOW (1971) determined that the double stranded form is subject to attack by ribonucleases even in 0.3M NaCl. The dsRNA is gradually digested over a period of time. It is therefore desirable in choosing the optimum ribonuclease concentration, to realise that the higher ribonuclease concentrations required to digest single stranded RNA completely, may also have the disadvantage of causing nicks in the phospho-diester



backbone of dsRNA, or actual degradation of the whole dsRNA molecule. LOVINY and SZEKELY (1973) actually used ribonuclease A to digest dsRNA from a fungal virus to analyze and fingerprint the sequence of nucleotides. They reported that the enzyme:substrate ratio needed for complete digestion was a 10:1, ribonuclease:RNA ratio in high salt. Initially in the experiments described in this chapter, 50  $\mu\text{g/ml}$  of ribonuclease A was used to digest 50  $\mu\text{g}$  of RNA in 1.5 ml of high salt solution. This represented a ribonuclease:RNA ratio of 1:1. This was reduced to 20  $\mu\text{g/ml}$  ribonuclease A for the same quantity of RNA, giving a ratio of 1:2.5. Since definite partial degradation of dsRNA has been observed at the ratio of 1:1 (LOVINY and SZEKELY, 1973) a reduced ratio of ribonuclease:RNA was finally adopted for the preparation of whole dsRNA. It is for this reason that a final concentration of 20  $\mu\text{g/ml}$  of ribonuclease A for the digestion at a concentration of 80  $\text{OD}_{260}$  nm units/ml (1:13) was adopted. At this concentration with the addition of ribonuclease  $T_1$ , it appears <sup>likely</sup> that all but a few small single nucleotide regions remain, but that the double stranded molecule is retained intact.

A survey of the literature reveals that little consideration has been given to the latter property, the ribonuclease:RNA ratio. Concentrations of ribonuclease A and the length of time and digestion temperature also differ. Concentrations of ribonuclease A range from 2  $\mu\text{g/ml}$  (JELINEK and DARNELL, 1972) to 50  $\mu\text{g/ml}$  (RYSKOV *et al.*, 1973).

Neither of the preparations of ribonuclease resistant RNA prepared by HAREL and MONTAGNIER, 1972 or JELINEK and DARNELL, 1972, used any  $T_1$  ribonuclease in their preparation. They make no remarks on the presence or absence of possible purine rich single stranded RNA contamination. The  $T_1$  ribonuclease concentration varies very little between the different workers. Slight variations in concentration of ribonuclease  $T_1$  are unimportant (WHITELAM and NAORA, unpublished observations), but the presence of this ribonuclease is important. Although the ribonuclease concentrations are given by each group of workers, no attention has been reported as being given to the actual concentration of RNA or the ribonuclease: RNA ratio. This is a vital consideration, for it is of prime concern that conditions of digestion do not allow dsRNA degradation or breaking of the backbone linkage, if further heat denaturation experiments which depend on intact dsRNA molecules are to be meaningful. The consideration of this ratio in this chapter has been to establish a standard condition for intact dsRNA preparation.

The effect of heat on the stability of the ribonuclease resistant material is an indication of dsRNA content. Heating the ribonuclease resistant RNA to 100°C for 10 min, should successfully separate any double strands. Rapid cooling in ice then prevents reannealing of the two strands of RNA. The difference in ribonuclease resistance before and after heating gives some measure of dsRNA content and these percentages are indicated in the section 2-3-3, as

described above. This property has been used here to identify dsRNA in the crude preparation. In the following chapter, a more detailed consideration of the properties of dsRNA and the heat denaturation profile will be given.

#### 2-4-2 *Differential Extraction from the Interface*

A lengthy discussion of the methods of extraction is not the subject of this thesis, and details of various methods of RNA extraction can be found in reviews on this subject, (e.g. STEELE and BUSCH, 1967 and references cited therein). Nevertheless, it was important to establish that the method of extracting whole RNA from the cell was not extracting a particular fraction or leaving dsRNA in the interface, which MONTAGNIER (1968) suggested was rich in this molecule.

Several factors determine the molecular species of RNA that is extracted from whole cells. Among these are the detergent used, and its concentration, the temperature of extraction, ionic strength and pH of the extraction medium. In the absence of detergent, nuclear material containing nuclear RNA has been reported as remaining at the interface after phenol extraction (SIBATANI, 1966; GEORGIEV, 1967). Differential extraction of a particular species of RNA is well documented. GEORGIEV (1962, 1967, 1972) details a method for the particular extraction of different classes of RNA, by varying the temperature of extraction. Other authors have demonstrated, by varying the detergent or concentration and temperature, the differential extraction of cytoplasmic and high molecular weight



nuclear RNA (COOPER and KAY, 1969). It might therefore have been likely that differential extraction of dsRNA may have occurred, as indeed was suggested by MONTAGNIER (1968). This possibility was investigated by rigorous treatment of the first phenol extraction interface, and while RNA was extracted from this interface, it did not appear to differ significantly in specific activity from the RNA isolated from the first aqueous supernatant after phenol-sodium dodecyl sulphate extraction, nor was the percentage of dsRNA of either fraction markedly different. It can be concluded that the particular conditions of extraction used in the isolation of RNA did not allow differential extraction of dsRNA under these circumstances.

#### 2-4-3 *Purification Procedures*

Various methods of purification of the dsRNA fraction exist. This step is particularly vital in the preparation of dsRNA, for without adequate purification no precise analysis of the fraction obtained could be achieved. The behaviour of the ribonuclease resistant fraction also provides information about dsRNA, and in fact the behaviour on chromatographs can be taken as a marker for double stranded character.

Various methods of separation have been used by different workers in attempts to partially purify the double stranded fraction. In early work on ribonuclease resistant fractions no purification of the fraction was attempted, (MONTAGNIER, 1968; COLBY and DUESBERG, 1969

TABLE 2-5

Author and Date	Source of Material	Prepn. RNA	Purification of RNA		Digestion conditions		Conc. of RNA	RNase: RNA ratio	Further Purification before analysis
			Protease	DNase	RNase A	RNase T <sub>1</sub>			
Stern & Friedman 1970, 1971	Burkitts Lymphoma	Phenol -SDS	-	20 µg/ml 30 min 37°C	12 µg/ml 60 min 23°C	2 µg/ml 23°C			None
Kimball & Duesberg 1971	Rabbit kidney Hela cells C.E.F.	Phenol -SDS	-	50 µg/ml 30 min 37°C	20 µg/ml	2 µg/ml			6% agarose gel
Harel & Montagnier 1972	Rat liver	Phenol -SDS	Was used concn. ?	Was used concn. ?	10 µg/ml 60 min 25°C	-			G-200 Sephadex to remove very small single nucleotides
Kronenberg & Humphreys 1972	Sea urchin embryos	Phenol -SDS	-	20 µg/ml 30 min 25°C	20 µg/ml 30 min 37°C	20 units/ ml			Cellulose stepwise elution
Jelinek & Darnell 1972	Hela cell nuclei	Phenol -SDS	-	-	2 µg/ml 30 min 37°C	-			Hydroxyapatite purification
Ryskov <i>et al.</i> , 1973	Ehrlich Ascites cells	3 Temper- ature phenol extrn.	200µg/ml 30 min 37°C	20 µg/ml 20 min 25°C	50 µg/ml 1 h 37°C	100 units/ ml			Sephadex G-75
Monckton 1971-1974	Rat liver and testes 1. Whole cells 2. Nuclei 3. Cytoplasm	Phenol -SDS	100µg/ml 37°C 1 h	20 or 50 µg/ml 37°C 30 min	20µg/ml 37°C 30 min	1 µg/ml	80 OD <sub>260</sub> units/ml	1:13	Sephadex G-50 G-200 Cellulose stepwise elution finally adopted

and FRIEDMAN, 1970). Heat denaturation profiles and base analyses were conducted on the material after ribonuclease digestion and subsequent phenol extraction. In view of the heterodisperse behaviour of the crude fractions, it is difficult to accept their measurements for particular double stranded properties, e.g. heat denaturation or base analysis. While these may be indicative of double stranded character and hence dsRNA, the measurements cannot be absolutely applied to dsRNA without further purification.

While purification itself is important, the method used is also important. Methods of separating small nucleotide fragments from true dsRNA after ribonuclease digestion and ethanol purification, depend upon different properties. Methods which separate molecules on account of molecular weight differences include molecular weight sieve chromatography, such as Sephadex or agarose and acrylamide gels. Other methods of separation include hydroxyapatite and cellulose chromatography. Where purification methods have been used by different workers, the choice in adopting that method has not always been clear. Table 2-5 contains a brief summary of the preparation and purification procedures adopted by workers dealing with ribonuclease resistant dsRNA fractions. KIMBALL and DUESBERG (1971), HAREL and MONTAGNIER (1972) and RYSKOV *et al.*, (1973), used molecular weight sieves, either agarose or Sephadex, to purify and remove single stranded contaminants. No details or comments on these methods were given. Of the other available methods, JELINEK and DARNELL



(1972) used hydroxyapatite, and in both the work of KRONENBERG and HUMPHREYS (1972) and the purification method described in this text, stepwise elution of cellulose was employed.

The Sephadex method was also employed in the present study. This method is a molecular sieve method, *viz* it separates molecules of RNA on the basis of their molecular weight and consequently size. This method has limitations and does not appear to be wholly suitable for the task, i.e. the isolation of an intact dsRNA preparation. Sephadex can successfully remove very small (1 or 2) nucleotide fragments, but is unsuitable, particularly for separation of molecules of double stranded RNA which may have larger single stranded RNA tails. The method is useful however in providing an indication of ribonuclease resistant RNA behaviour, and some indications of the range of molecular sizes.

The hydroxyapatite method for separating single and double stranded polyribonucleotides was originally developed and reported by BERNARDI (1969). The technique involves the principle that single stranded, random coiled polynucleotides are eluted at lower phosphate molarities than double stranded rigid polynucleotides from a column of hydroxyapatite crystals. In general, single stranded RNA's elutes in a lower molarity phosphate buffer (0.15 M) while the double stranded molecule tends to elute in a higher concentration (0.20-0.22 M). There are various anomalies with the

method, however, and poly(A) and poly(I) only elute at higher molarities. The separation is not absolutely defined, and as with Sephadex separation, this method does not allow detection between possible variations, e.g. the double stranded molecule with a single stranded attached region. The hydroxyapatite method has been used to separate double stranded viral RNA's. It was also used recently by JELINEK and DARNELL (1972) but no comment was made as to the success of this technique in purification of dsRNA.

A better method of separating a purified dsRNA fraction, was to employ a technique of stepwise elution from a cellulose column. The technique exploits the change in chemical activity of nucleic acids in buffers containing variable amounts of alcohol and was employed first by BARBER (1966) to separate tRNA from ribosomal RNA. More specifically FRANKLIN (1966) employed the technique to separate truly double stranded forms from ribosomal RNA. He clearly demonstrated that the tRNA elutes in the 65% TSE buffer:35% ethanol fraction, ribosomal RNA eluted in the 85% TSE buffer:15% ethanol. The dsRNA fraction then elutes in the buffer alone. The mechanism of actual separation of the different classes of RNA is not entirely understood. It is apparent that fractionation of classes of RNA depend on differences in molecular structure, whereas fractionation on Sephadex or agarose, depends on molecular weight differences. The exact interaction of structure with the cellulose is not known. The cellulose method provides an

accurate and reproducible method of separation of the classes of RNA. It is this separation on the basis of structure of the molecule which allows cellulose chromatography to stand apart as the ideal method of separation of a truly dsRNA.

One other feature commends cellulose as a method of preparing the dsRNA fraction. Sephadex separation has some limitations on the amount of digested RNA that can be applied. Fairly large Sephadex columns were used (2.5 x 35 cm) but the amount of RNA which could be applied to achieve ideal peak separation was 20 OD<sub>260</sub> nm units/ml. This places a practical restriction on the preparation of large quantities of purified dsRNA for analysis, bearing in mind that dsRNA represents such a small percentage of the total extractable RNA. In contrast, although the size of the column for identification of dsRNA is small, a larger cellulose column can be used for the preparation of dsRNA for further experimentation. The larger column can be loaded with as much as 50 mg of ribonuclease resistant RNA and a yield of 450 µg of purified third peak material can then be obtained.

A detailed investigation of the pooled fraction of the third peak fractionation will be the subject of the investigations of the next chapter. Preliminary analysis suggests that the third peak fraction definitely contains the dsRNA, having the characteristic susceptibility to further ribonuclease digestion after heat denaturation. Other evidence in support of the cellulose characterization method, such as isolating the double stranded molecule, is seen in that two well known double stranded forms, reovirus dsRNA and synthetic double stranded poly(I).poly(C), both chromatograph in the



third peak region. In addition, the denatured form of reovirus RNA then chromatographs in the second peak region. These independent investigations not only lend support to the suitability of the cellulose technique, but also attest to the purity of the dsRNA fraction which can be obtained. The investigations described in the next chapter will describe the detailed properties of the third peak fraction and independently confirm the advantage of the cellulose technique and the purity of the dsRNA fraction.

The first aim of the experiments described was to confirm the double stranded character by its heat denaturation curve, cesium sulphate density gradient profile, immunodiffusion behaviour and base composition. In particular a biphasic heat denaturation profile is the most reliable indication of a double helical structure. The gel electrophoretic behaviour was also investigated to determine the size and heterogeneity of dsRNA molecules. Finally, experiments were carried out to confirm that the double stranded structure certainly consists of RNA molecules alone, and does not involve a DNA-RNA hybrid structure.

It has already been noted in Chapter II that the early dsRNA fractions isolated by other workers were not sufficiently purified. Having established a technique of purification, a full characterization of this species of RNA is required for a better understanding of the function and biological significance of this new type of RNA.

## CHAPTER III - PROPERTIES OF dsRNA: CONFORMATION OF DOUBLE STRANDED CHARACTER

### 3-1 INTRODUCTION

Having isolated an RNA fraction by cellulose chromatography, which indicated by virtue of its heat denaturation-enzyme susceptibility behaviour that it appeared to be double stranded, it was important to confirm that this fraction represented a truly double stranded form. The first aim of the experiments described was to confirm the double stranded character by its heat denaturation curve, caesium sulphate density gradient profile, immunodiffusion behaviour and base composition. In particular a biphasic heat denaturation profile is the most reliable indication of a double helical structure. The gel electrophoretic behaviour was also investigated to determine the size and heterogeneity of dsRNA molecules. Finally, experiments were carried out to confirm that the double stranded structure certainly consists of RNA molecules alone, and does not involve a DNA-RNA hybrid structure.

It has already been noted in Chapter II that the crude dsRNA fractions isolated by other workers were not sufficiently purified. Having established a technique of purification, a full characterization of this species of RNA is required for a better understanding of the function and biological significance of this new type of RNA.

### 3-2 MATERIALS AND METHODS

Generally, the materials and methods are identical with those described in Chapter II. In this section only materials and methods which apply specifically to this chapter will be given.

#### 3-2-1 *Materials*

Additional chemicals required for analysis of methods were as follows. Poly(I).poly(C) was obtained from PL Biochemicals. Acrylamide and N,N'-methylene bis-acrylamide were purchased from Eastman Organic Chemicals. The caesium sulphate used was obtained from Merck and was spectrophotometrically pure. Antiserum prepared specifically against poly(I).poly(C) was a gift of Dr. R.I.B. Francki, of the Waite Agricultural Research Institute of South Australia.

#### METHODS

#### 3-2-2 *Pooling of Fractions with Subsequent concentration and/or Dialysis of Third Peak Cellulose chromatographed material*

For analysis purposes the material eluted in third peak of the cellulose column, was concentrated and/or dialyzed or precipitated after concentration and redissolved in the particular buffer required for analysis. Fractions were eluted in the column buffer TSE, which has a lower concentration of NaCl (0.1M) than is optimum (0.3M) for the stability of dsRNA. Consequently the concentrate of the fractions from the third peak was, after pooling, adjusted to 0.3M NaCl by the addition of concentrated NaCl.



The concentration and/or dialysis of these fractions depended upon the amount of RNA contained in the third peak after cellulose chromatography, it also depended on the type of analysis to be carried out, e.g. for heat denaturation, material was isolated from the third peak after chromatography on a small cellulose column, and concentration was unnecessary as the total volume of the pooled fractions was 10-12 ml. In this case the pooled material was dialyzed directly against the required solution (0.1 x SSC or 2 x SSC), because 10-12 ml was ideal for 10-12, 1 ml heat denaturation fractions. Dialysis was carried out at  $-4^{\circ}\text{C}$  with three changes of solution. The dialysis solution was at least 100 times the volume of the sample.

For gel electrophoresis, base analysis or caesium sulphate density gradient analysis or immunodiffusion, it was important to concentrate the third peak fraction as much as possible to enable further analysis, as these techniques require application of small concentrated samples. Quantitative yields of third peak material were desirable to avoid loss of valuable material, but generally the concentration of dsRNA in the third peak pooled fractions was insufficient to allow direct ethanol precipitation of radioactivity labelled dsRNA. A method of concentration was therefore adopted to allow concentration of the dsRNA from the larger volumes of pooled fractions. These techniques were as follows:

- (1) A 40 ml capacity AMICON diaflo ultrafiltration apparatus fitted with a filter membrane (UM 10) capable of retaining all molecules of larger than 10,000 molecular weight, was employed.

This apparatus concentrated to volumes of 1-2 ml without necessitating change in the ionic conditions of the buffer in which the RNA is dissolved. This small volume was then either dialysed against the requisite buffer or concentrated further by the technique described below.

(2) The pooled fractions (or AMICON concentrated fraction) was collected in dialysis tubing, preferably of small diameter and (8mm) FICOLL (SIGMA) (a highly polymerized sugar which concentrated by absorbing the buffer solution through the dialysis tubing) was applied. This procedure effectively concentrates the RNA preparation, with careful manipulation, to volumes as small as 20-50  $\mu$ l without appreciable losses. Care must be taken to avoid contamination during handling procedures.

For larger volumes of pooled fractions, e.g. the large cellulose column (usually unlabelled material) AMICON concentration to 2-3 ml followed by ethanol precipitation at  $-20^{\circ}\text{C}$  overnight, gave reproducible reasonable yields of purified dsRNA.

### 3-2-3 *Heat Denaturation*

Aliquots of labelled dsRNA dissolved in 1 ml low salt solution ( $0.1 \times \text{SSC}$ ), were heated gradually over a  $40-100^{\circ}\text{C}$  temperature range. This heating was conducted in a HAAKE constant temperature bath where the rise in temperature was regulated to  $2^{\circ}\text{C}$  per min. The RNA samples were contained in pyrex tubes, carefully sealed with two layers of plastic

(Glad-wrap) and secured with rubber bands. Tubes were removed at successive temperatures and plunged immediately into ice, the salt concentration was adjusted to 0.3M NaCl by the addition of 20 x SSC to 2 x SSC, and were subsequently digested at 37°C with pancreatic ribonuclease A (20 µg/ml). The tubes were cooled and after addition of 50 µg of unlabelled rat liver RNA as a carrier, the RNA was precipitated with trichloroacetic acid and filtered, dried and counted as described in section 2-2-10.

The heat denaturation temperature ( $T_m$ ) was obtained from the inflexion point of this biphasic curve. The  $T_m$  is ionic concentration dependent and the heat denaturation was alternatively conducted with initial temperature incubations in which the dsRNA was dissolved in 2 x SSC (high salt solution).

#### 3-2-4 *Base Analysis*

$^{32}\text{P}$  labelled material isolated from the third peak of cellulose chromatography was analyzed for base analysis.

To the labelled RNA after pooling and concentration, 100 µg of unlabelled rat liver cellular RNA was added. These RNAs were precipitated with an equal volume of ice-cold 10% trichloroacetic acid, centrifuged at 2,500 rpm on the bench centrifuge, washed and recentrifuged twice with 5 ml ice-cold trichloroacetic acid and finally 5 ml of cold absolute ethanol and dried. 0.2 ml of 0.3N KOH was added to the precipitate and incubated for 18 h at 37°C. This solution was neutralized with 1N perchloric acid (adjusted to pH 7.2) then frozen. The



frozen precipitate was centrifuged 2,500 rpm for 5 min on the bench centrifuge, and the supernatant carefully evaporated using a vacuum rotary evaporator. Water (20  $\mu$ l) was added to dissolve the mononucleotides remaining.

These mononucleotides were spotted onto thin layer cellulose plates. (DS Fertigplatten Cellulose, F. Merck). Development was carried out with a mixture of 66% isobutyric acid and 1% ammonia dissolved in distilled water for 7 h, followed by a second development with a mixture of 80%  $(\text{NH}_4)_2\text{SO}_4$  (saturated solution), 18% sodium acetate, and 2% isopropanol for 8 h. The separated mononucleotides were then removed from the dried plate by scraping the cellulose with a fine spatula and individually collecting each labelled mononucleotide. These were then eluted with 0.1N HCl and aliquots of the centrifuged eluants of each nucleotide dried and counted as previously described, using 10 ml PPO-POPOP toluene scintillant.

#### 3-2-5 *Caesium Sulphate Density Gradient Fractionation*

Caesium sulphate density gradient centrifugation was performed as follows: The particular dsRNA, RNA or DNA sample was dissolved in 3.8 ml of 50 mM Tris-HCl (pH 7.4) buffer containing sufficient  $\text{Cs}_2\text{SO}_4$  to give a final average density of 1.58 g/ml. The solutions were covered with paraffin and sealed. Centrifugation was at 20°C and 31,500 rpm for 72 h in a Spinco SW 50.1 rotor. Gradients were fractionated by collecting drops from a needle held in place piercing the bottom of the tube. The flow of drops from the needle was controlled by a paraffin injection apparatus, fitted with a

micrometer. Ten-drop fractions were collected, except that every sixth fraction contained five drops. 50  $\mu$ l aliquots were measured from these smaller fractions, using a calibrated 50  $\mu$ l glass micropipette, and weighed to 5 decimal places. Refractometric measurements were made as a confirmatory check. The optical density at 260 nm was determined for each of the 10 drop fractions after diluting in 1 ml  $H_2O$ .

#### 3-2-6 *Immunodiffusion Technique*

Immunodiffusion plates were prepared according to the method described in FRANCKI and JACKSON (1972) and 3 mm holes cut in a typical serological pattern. Samples of different types of RNA were applied in the outer wells (10  $\mu$ l) and the antiserum (10  $\mu$ l) was applied to the centre well. The plates were allowed to develop at room temperature for 48 h and examined every 12 h. The plates were photographed using a dark field illumination, each plate being covered with distilled water to reduce shine effects.

#### 3-2-7 *Gel Electrophoresis*

Gel electrophoresis was carried out using 10% polyacrylamide gels made as follows:

##### Solutions

- (1) 13 mls of stock solution - 15% acrylamide  
0.4% bis-acrylamide
- (2) 2 mls of 10 x E buffer (concentrated gel buffer)  
(E buffer = 40 mM Tris HCl, 60 mM Sodium Acetate 3 mM EDTA pH 7.2)
- (3) 5 mls distilled water

(4) 70  $\mu$ l of N,N,N'N'-tetramethylethylene diamine (TEMED)

(5) 0.45 mls of freshly prepared 10% ammonium persulphate.

The first four solutions were mixed in a 250 ml side arm flask. The mixture was degassed by evacuation and ammonium persulphate was then added and swirled gently. The gel mixture was rapidly transferred to gel tubes (0.8 mM x 10 mm) and allowed to set for 1-2 h. 50  $\mu$ l of water was layered onto the surface of the gel to provide a flat surface for sample application.

Gels prepared for use in this way often have a high background absorbance at 254 nm which interferes with optical density scanning. This background can be reduced by pre-electrophoresing gels for 1 h (6 mA/gel) but even better results are achieved by soaking the preformed gels in E buffer containing 0.5% sodium dodecyl sulphate at 4°C for at least 24 h.

Gel electrophoresis was conducted at 4°C at 6 mA/gel for 150 min for 10% gels. The buffer used was E buffer (described above) containing 0.5% sodium dodecyl sulphate.

Gels were scanned after electrophoresis with an ISCO UA4 absorbance monitor fitted with a linear gel transport. Gels where necessary were then removed and sliced into 1 or 2 mm slices, using a MICKLE gel slicer, incubated with 0.5 ml of NCS solubilizer (Amersham/Searle) at 37°C for 12 h and counted in PPO-POPOP toluene scintillant.



### 3-2-8 *Labelling of both DNA and RNA in Regenerating Livers*

Rats were partially hepatectomized according to the previously described method (HIGGINS and ANDERSON, 1931). [ $^3\text{H}$ ] orotic acid (300  $\mu\text{Ci}$ /head) was injected intraperitoneally <sup>12</sup>~~48~~ h after the operation <sup>to label the DNA</sup> and a further 300  $\mu\text{Ci}$  was injected intraperitoneally <sup>48</sup>~~12~~ h after the administration of the first injection of isotope <sup>to label the RNA</sup> (BUCHER, 1963).

Alkaline hydrolysis of the material isolated from the third peak of cellulose and pooled and concentrated as described above, was carried out with 0.3M or 0.5M potassium hydroxide at 37°C for 18 h. Samples for heat denaturation were denatured as described in section 2-2-12 previously. Each hydrolyzed sample was then neutralized with 0.3N or 0.5N HCl and 50  $\mu\text{g}$  of carried RNA added, filtered and precipitated with ice-cold 5% trichloroacetic acid, filtered, washed twice with ice-cold 5% trichloroacetic acid and ethanol, dried and counted as described previously (section 2-2-10).

## 3-3 RESULTS

### 3-3-1 *Heat Denaturation*

The main argument for suggesting that the material eluted in the third peak on cellulose chromatography is dsRNA is that it behaved as a base-paired double stranded molecule when subjected to heat denaturation treatment.

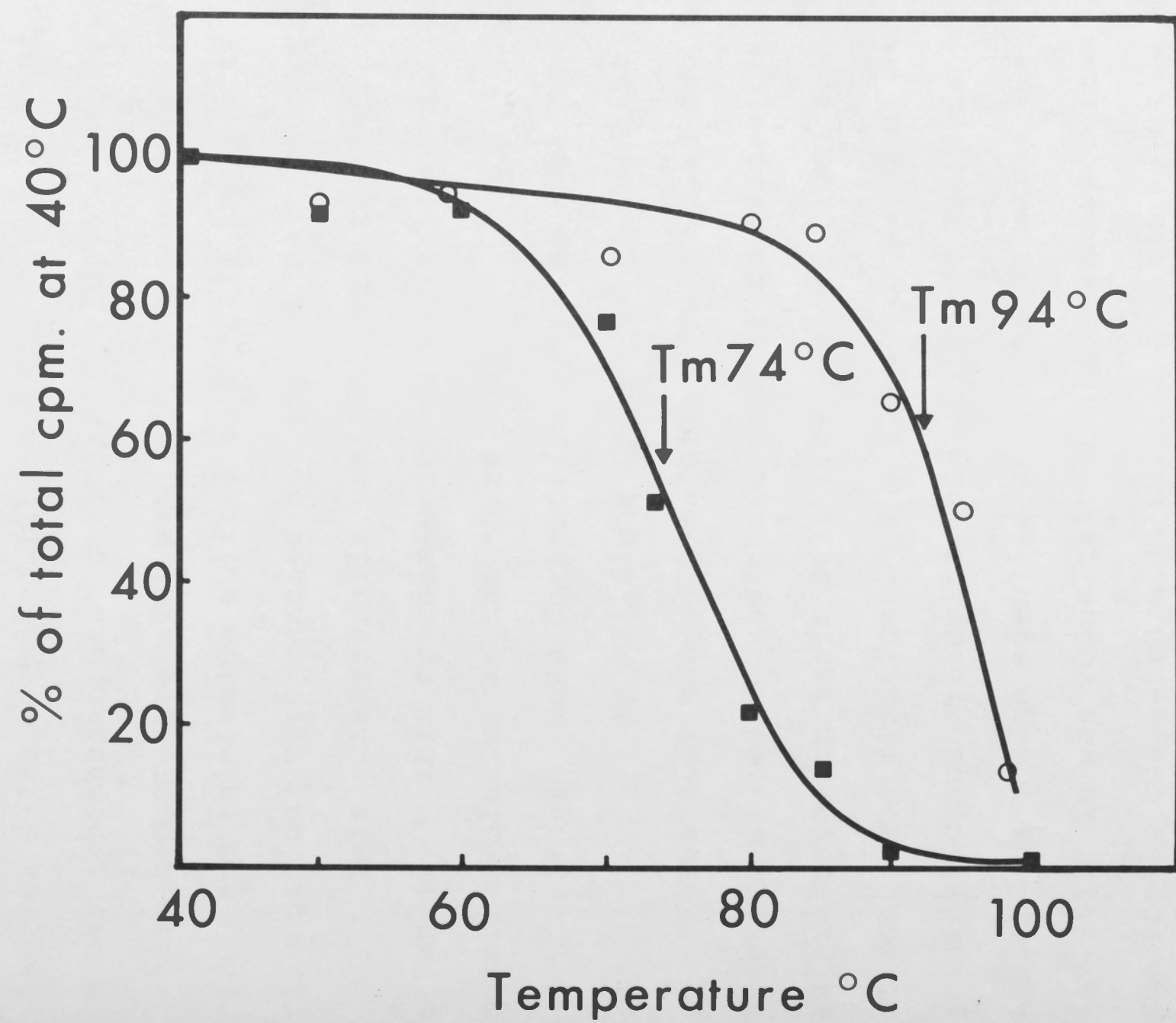
Whole cell RNA labelled with [ $^3\text{H}$ ] orotic acid for 60 min was digested with ribonucleases and purified by

FIGURE 3-1    Heat denaturation profile of  $^3\text{H}$ -  
labelled rat liver dsRNA

dsRNA in the third peak from cellulose chromatography of ribonuclease resistant RNA prepared from  $^3\text{H}$ -labelled cellular RNA was heat denatured in 0.1 x SSC or 2 x SSC, as indicated. The ribonuclease sensitivity at various temperatures were measured by ribonuclease A digestion in 2 x SSC (20  $\mu\text{g/ml}$ , 37°C, 30 min) and the acid precipitable radioactivity remaining expressed as a percentage of the total radioactivity at 40°C.

○ ——— ○ , Incubation in 2 x SSC

■ ——— ■ , Incubation in 0.1 x SSC





cellulose chromatography as described in Chapter II. The material eluted from the third peak on cellulose was pooled and each of two halves dialysed against low and high salt solutions (0.1 x SSC and 2 x SSC). The heat denaturation curves of this material were analyzed as described under section 3-2-3. The result is illustrated in Figure 3-1 and shows a biphasic heat denaturation curve. The heat denaturation temperature ( $T_m$ ) was 74°C at a concentration of 0.1 x SSC, and this value shifted to 94°C if the initial temperature incubations were carried out in high salt concentration (2 x SSC).

This substantially confirms that the structure is double stranded. This ~~transcription~~ curve was observed only with material eluting in the third peak.

#### 3-3-2 *Base Composition of Third Peak Fraction Obtained by Cellulose Column Chromatography*

The base composition of a  $^{32}\text{P}$ -labelled double stranded fraction eluted in the third peak of cellulose chromatography is presented in Table 3-1. The figures given are an average of four determinations. The figures show equal <sup>values</sup>~~volumes~~ for guanidylic and cytidylic acid content. The material is characterised by a high adenylic acid content and a low G + C content (37.3%). It was found that the high adenylic acid content was due to the coexistence of poly(A) in this fraction and this observation will be more fully explained in Chapter VI, where the relationship of dsRNA to poly adenylic acid is discussed.

The actual content of poly(A) in the third peak, determined by several methods, (see Chapter VI) was 12% of the total radioactivity of the third peak fraction. If

TABLE 3-1

Base composition of  $^{32}\text{P}$ -labelled ribonuclease resistant whole liver cellular RNA isolated from the third eluate of cellulose column chromatography. The values shown are the average of three determinations. See Chapter VI for details of correction for poly(A) content.

Base composition (moles percent)					G + C content (%)	G/C	A/U
	A	G	C	U			
Measured	37.5 $\pm$ 1.7	18.4 $\pm$ 1.2	18.9 $\pm$ 1.5	25.0 $\pm$ 1.8	37.3	0.97	1.50
Corrected	28.2	21.2	21.8	28.8	43.0	0.97	0.98

the base composition is corrected for 12% poly(A) content, the corrected values for G + C content is 43.0% and for adenylic acid 28.2%. The value for adenylic acid now closely compares with the corrected value of uridylic acid and  $A/U$  and  $G/C$  ratios are both nearly equal to 1. This provides additional evidence for the base paired structure of dsRNA eluting for the third peak of cellulose columns.

### 3-3-3 *Relationship of G + C Content and T<sub>m</sub> Values*

The relationship of denaturation temperatures of several known viral dsRNA's to their respective known G + C content is shown in Figure 3-2. The values and references are given in Table 3-2. Using this relationship, and the corrected values of G + C, a value of 95°C is the expected T<sub>m</sub> of rat liver dsRNA at high salt concentrations. This is consistent with the value of 94°C obtained by the heat denaturation method.

### 3-3-4 *Caesium Sulphate Density Gradient Centrifugation*

The material isolated from the third peak of cellulose chromatography of ribonuclease digested unlabelled cellular RNA was pooled and concentrated as described above. This material was analyzed for its behaviour on caesium sulphate density gradients. The results <sup>obtained in the preliminary experiment are</sup> ~~of this analysis is~~ presented in Figure 3-3, and indicate the presence of a broadly dispersed peak lying between the markers for DNA (salmon sperm DNA density as analyzed on identical gradient 1.425) and Hn RNA, representing a single stranded RNA (analyzed on similar gradient, major peak density 1.62). <sup>Further analysis is required for a</sup> ~~The position of reovirus~~



FIGURE 3-2    The relationship between heat denaturation temperature ( $T_m$ ) and the G + C content of various dsRNAs

The ordinate indicates heat denaturation temperature and the abscissa, the G + C content. Values for  $T_m$  and G + C, the abbreviations for dsRNA types, and their respective origins, are indicated in Table 3-2.

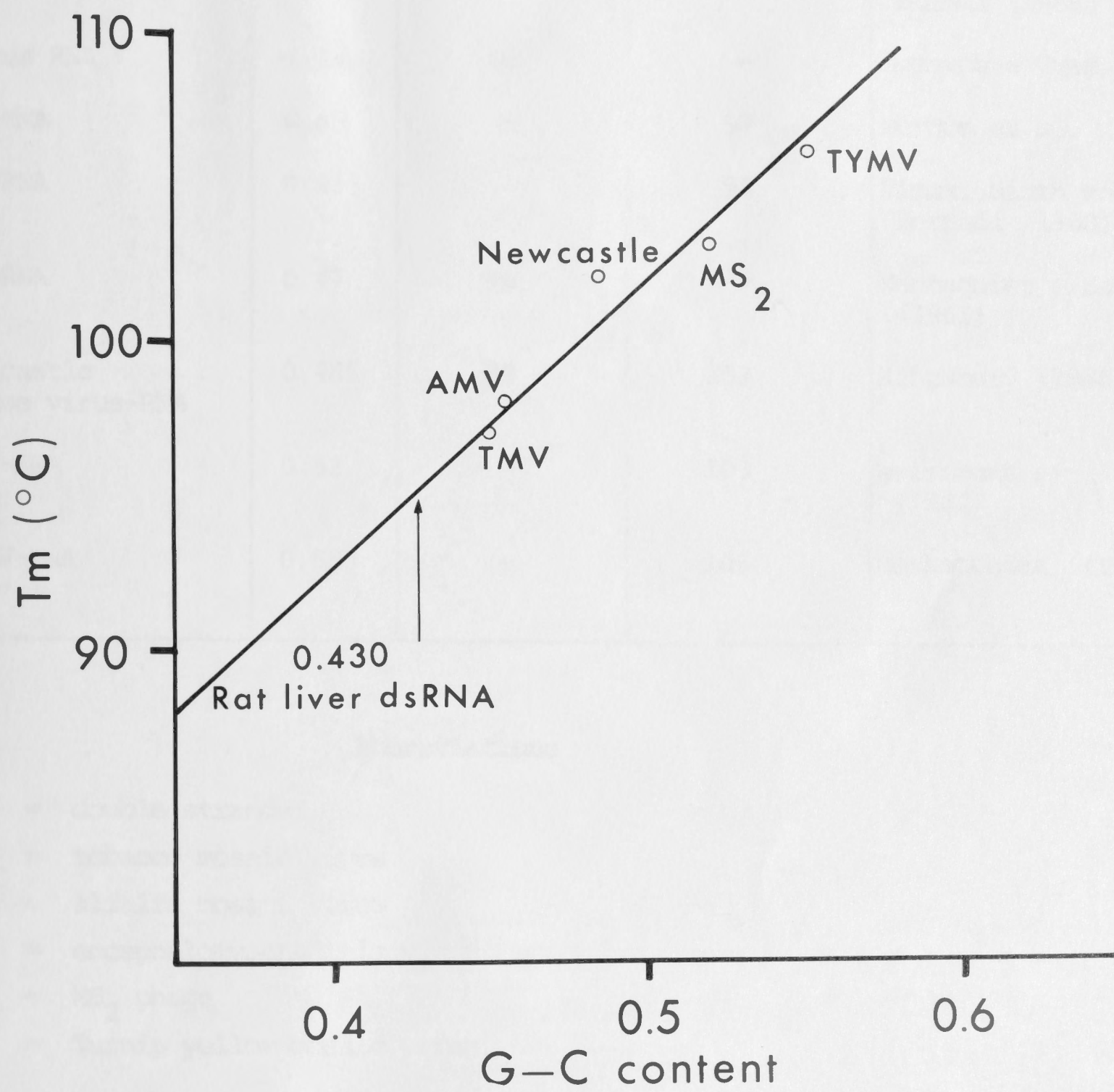


TABLE 3-2

	G + C content	T <sub>m</sub> (Hyper- chromicity)	T <sub>m</sub> (Ribonuclease resistance)	Reference
1. Wound Tumour virus RNA	0.38	90	-	Gomatos & Tamm (1963)
2. Rice dwarf virus RNA	0.438	95	-	Miura, Kimura & Suzuki (1966)
3. Reovirus RNA	0.44	93	-	Gomatos & Tamm (1963)
4. dsTMV-RNA	0.45	-	97	Burdon <i>et al.</i> (1964)
5. dsAMV-RNA	0.455	-	98	Pinck, Hirth and Bernadi (1968)
6. dsEMC-RNA	0.47	96	-	Montagnier & Sanders (1963)
7. ds Newcastle disease virus-RNA	0.485	98	102	Kingsbury (1966)
8. ds MS <sub>2</sub> -RNA	0.52	-	103	Weissmann <i>et al.</i> , (1964)
9. ds TYMV-RNA	0.553	-	106	Bockstahler (1967)

## Abbreviations

- ds = double stranded  
 TMV = tobacco mosaic virus  
 AMV = alfalfa mosaic virus  
 EMC = encephalomyocarditis virus  
 MS<sub>2</sub> = MS<sub>2</sub> phage  
 TYMV = Turnip yellow mosaic virus

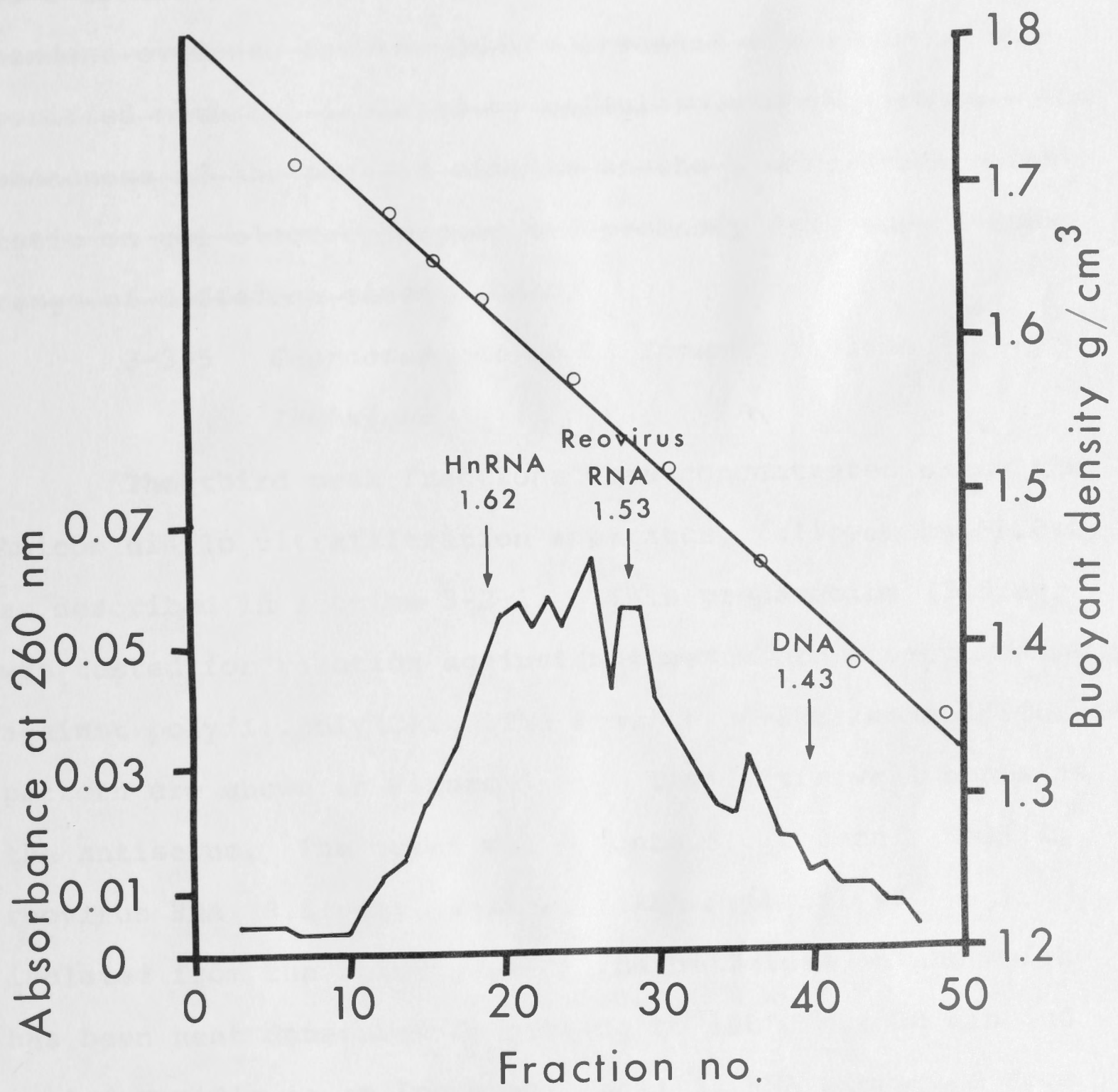


FIGURE 3-3    Caesium sulphate density gradient profile of  
dsRNA isolated from rat liver whole cellular  
RNA

dsRNA isolated from the third peak of cellulose chromatography of ribonuclease resistant RNA prepared from cellular RNA, was dissolved in 50 mM Tris-HCl, pH 7.4, containing sufficient caesium sulphate to give a final average buoyant density of 1.58 g/ml. Centrifugation at 20°C was for 72 h at 31,500 rpm. Ten and five drop fractions were collected from a hole pierced in the base of the tube, and the absorbance at 260 nm and the buoyant density determined from the ten and five drop fractions respectively. The position of salmon sperm DNA (1.43 g/ml), the single stranded RNA (HnRNA) from rat liver (1.62 g/ml) and reovirus dsRNA, (1.53 g/ml) are indicated.

\_\_\_\_\_ , Absorbance at 260 nm

○————○ , Buoyant density (g/ml).



definite conclusion.

~~RNA was also similarly analyzed and has a density of 1.53.~~

~~The finding that the peak of rat liver material lies also in this region and between the values for DNA and single stranded RNA is in agreement with the expected properties of a double stranded molecule of RNA and is further independent evidence for the double stranded character of the purified material isolated by cellulose chromatography. The broadness of the peak is similar to the observed characteristic on gel electrophoresis and probably indicates a broad range of different sized dsRNAs.~~

### 3-3-5 Characterization by Immunodiffusion

#### *Technique*

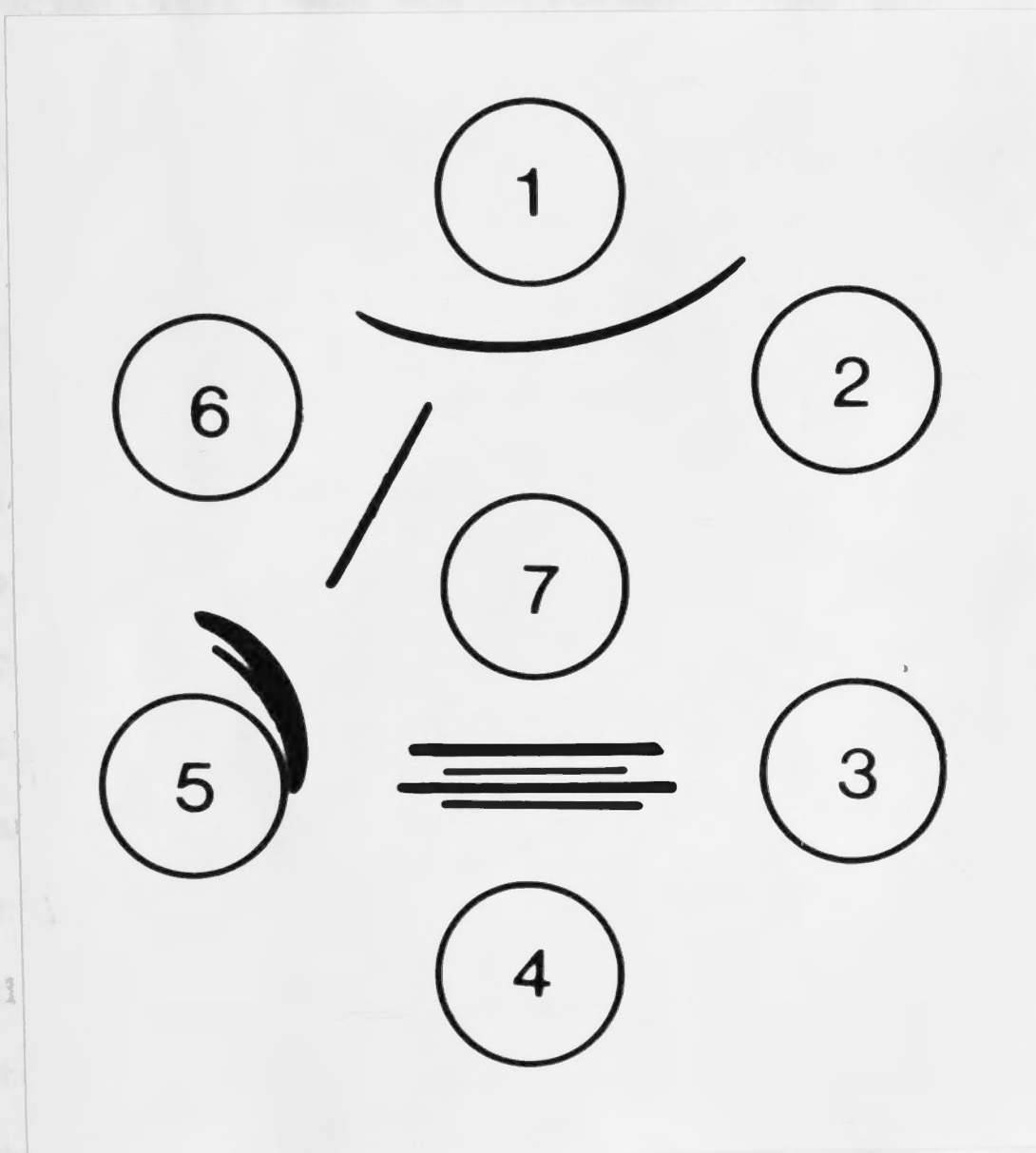
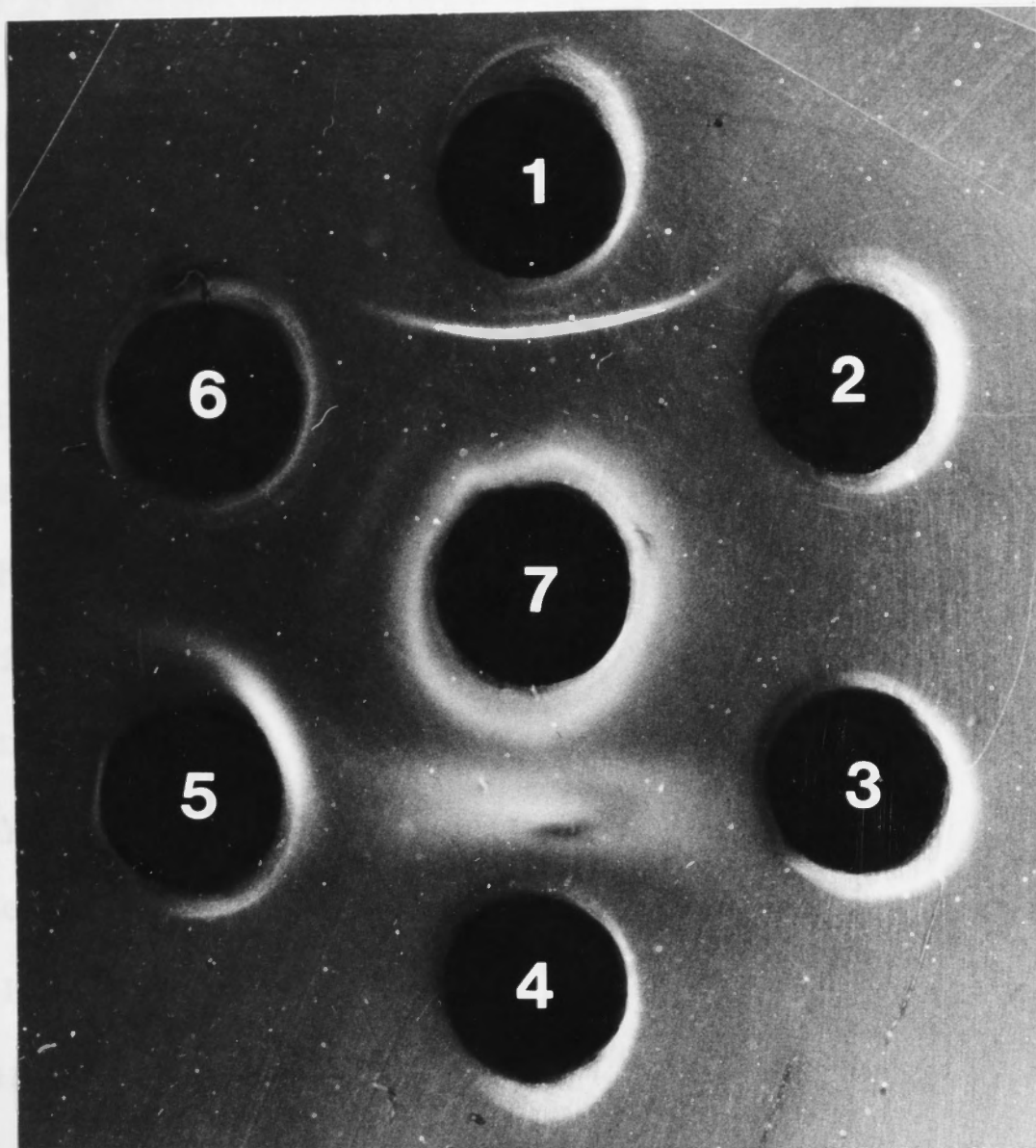
The third peak fractions were concentrated using the Amicon diaflo ultrafiltration apparatus, followed by FICOLL, as described in section 3-2-1. This preparation (2.5  $\mu$ g) was tested for reaction against antiserum prepared specifically against poly(I).poly(C). The results of the immunodiffusion pattern are shown in Figure 3-4. The centre well contains the antiserum. The outer wells contain, in turn: Well 1, reovirus RNA (8.5  $\mu$ g); Well 2, dsRNA material (2.5  $\mu$ g), isolated from the third peak of the cellulose column which has been heat denatured by heating to 100°C for 10 min and cooled rapidly in an ice bath; Well 3, RNA extracted from whole liver cells (10  $\mu$ g); Well 4 and 5 (1  $\mu$ g) two different batches of poly(I).poly(C); Well 6, also dsRNA but not heat denatured (2.5  $\mu$ g).

The precipitation activity of the antiserum used



FIGURE 3-4    Two dimensional immunodiffusion of antiserum  
to poly(I).poly(C) with various nucleic acid  
preparations

Antiserum was placed in the centre well. Outer wells contained 10  $\mu$ l of preparation in the amounts indicated; Well 1, reovirus RNA, (8.5  $\mu$ g); Well 2, dsRNA material isolated from the third peak of the cellulose column which had been denatured by heating to 100°C for 10 min and cooled rapidly in the ice bath (2.5  $\mu$ g); Well 3, RNA extracted from whole liver cells (10  $\mu$ g); Well 4 and 5, two different batches of poly(I).poly(C) (1  $\mu$ g); Well 6, the same as Well 2, i.e., dsRNA prepared from rat liver cells but not heat denatured. The "halo" around the antiserum well was non-specific and is believed to be due to diffusion of serum proteins. The 'shine' on the right sides of each well is due to the photographic technique.



here to the double stranded structure of RNA was clearly observed with poly(I).poly(C) and reovirus RNA. Two different preparations of poly(I).poly(C) used in this experiment gave precipitation lines but in a slightly different manner. Commercial batches of poly(I).poly(C) differ in their constitution, nucleotide length etc., and observed differences are probably therefore due to the variation in size and homogeneity of the preparations of poly(I).poly(C). The double stranded fraction eluted from the third cellulose peak definitely reacted to the antiserum (Well <sup>6</sup>~~1~~). However, the precipitation line was totally absent when an equivalent amount of the same preparation had been heat denatured (Well <sup>2</sup>~~1~~). This is strong, independent evidence confirming that the third peak RNA was double stranded. Any visible precipitation was not observed with whole cell RNA or DNA.

### 3-3-6 *Size*

The majority of acid insoluble material in ribonuclease resistant RNA eluted from G-50 Sephadex as a broad peak in the region with marker tRNA (Figure 2-4). This indicated that the ribonuclease resistant RNA was heterogeneous in size. A similar conclusion was obtained with purified dsRNA, although the dispersion was not as broad. dsRNA from whole cells, purified through the cellulose column, was applied to 10% polyacrylamide gels and electrophoresed for 150 min. Figure 3-5 illustrates the profile obtained with <sup>32</sup>P-labelled cellular dsRNA. The gel electrophoretic pattern obtained demonstrates that the dsRNA was composed of molecules of two distinct types. The first dsRNA peak was rather broad, high

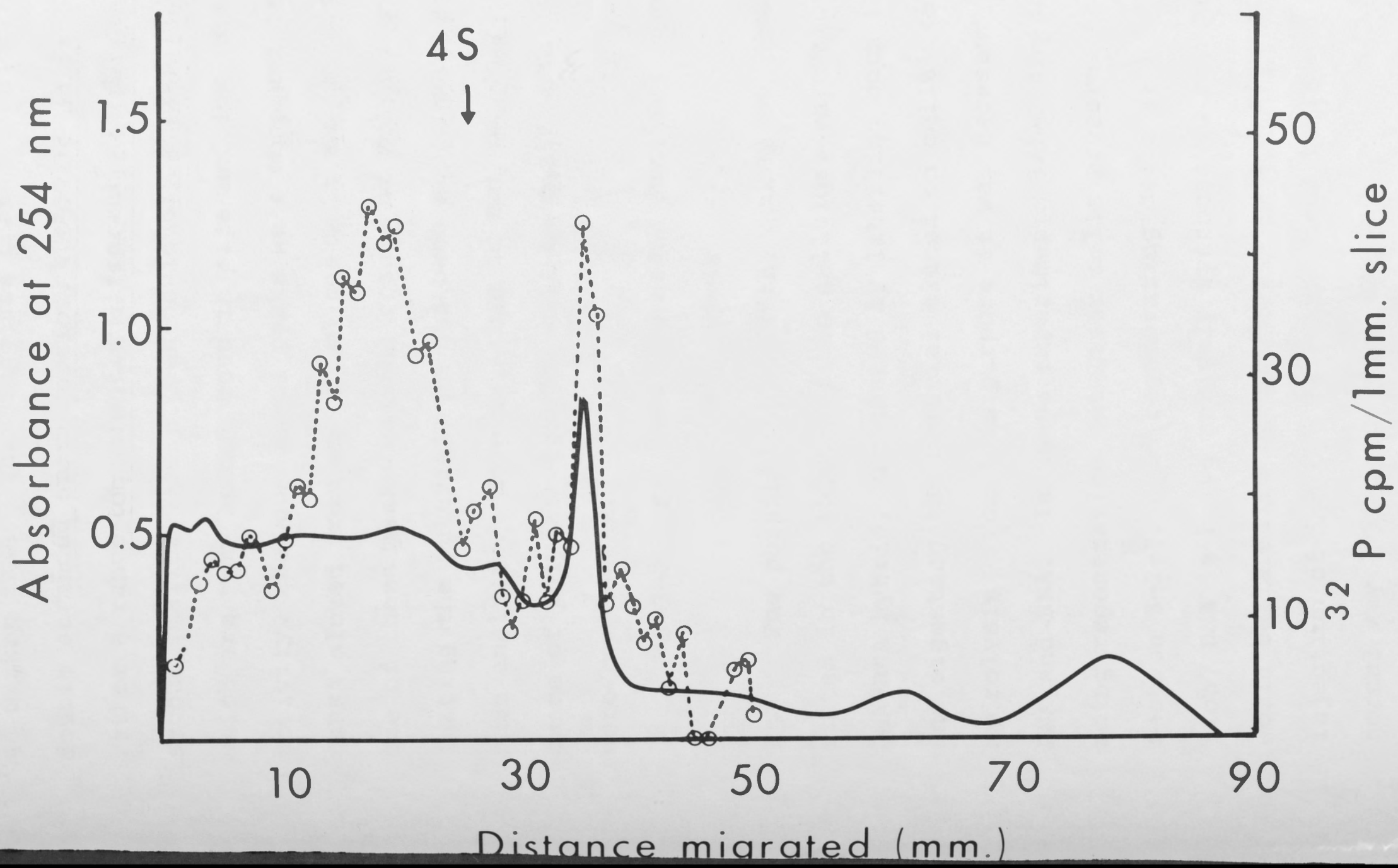


FIGURE 3-5 Migration of dsRNA isolated from  $^{32}\text{P}$ -  
labelled whole cell RNA in 10% poly-  
acrylamide gels

dsRNA was prepared from the third peak cellulose chromatographed material of ribonuclease resistant RNA from  $^{32}\text{P}$ -labelled rat livers, and analyzed on polyacrylamide gels. Gels were scanned for absorbance at 254 nm, sliced into 1 mm slices and assayed for radioactivity. The position of rat liver tRNA applied as a marker (4S) is indicated.

\_\_\_\_\_, Absorbance at 254 nm

○.....○, Radioactivity/mm slice.



in specific activity, and migrated slightly slower than the 4S marker tRNA. This suggests that the majority of the double stranded species are not absolutely homogeneous, but rather a range of slightly different sized dsRNAs. The second smaller, sharp peak obviously represented molecules which are very homogeneous in size and low in specific activity. Because these peaks were observed from purified dsRNA eluted from the third peak, it may be suspected that one of these peaks was the result of poly(A) segments contaminating the material, as has been mentioned in discussing base analysis. However, this is not the case. The significance of poly(A) segments will be dealt with in Chapter VI more completely.

### 3-3-7 *The Purified dsRNA Fraction does not contain a DNA-RNA Hybrid*

The possibility that the double stranded material eluted in the third peak on cellulose chromatography is a DNA-RNA hybrid, was tested by labelling both the DNA and RNA and digesting the material eluted on cellulose by alkaline hydrolysis. For this purpose it was necessary to label both DNA and RNA. In these experiments labelling was carried out using regenerating rat liver cells prepared as described in section 3-2-<sup>8</sup>~~7~~. In regenerating cells 48 h after the operation, DNA is being actively synthesized and consequently would be labelled by an administration of isotope. A second injection of isotope is mainly used to label the RNA in the normal way. The procedure adopted for purification was



TABLE 3-3

Effect of KOH hydrolysis on  $^3\text{H}$ -labelled RNA isolated from the third peak of a cellulose column. RNA was prepared as dsRNA, but regenerating livers were labelled for DNA and RNA<sub>A</sub> with [ $^3\text{H}$ ]uronic acid. In some experiments KOH hydrolysis was preceded by heat denaturation or heat denaturation and deoxyribonuclease. Treated and untreated RNA was trichloroacetic acid precipitated and filtered and counted, as described in 2-2-10. 3500 cpm of  $^3\text{H}$ -labelled RNA was added to each of five tubes.

Treatment	cpm remaining
1. No treatment	3,500
2. 0.3N KOH hydrolysis, 37°C 18 h	8
3. 0.5N KOH hydrolysis, 37°C 18 h	7
4. Heat denaturation, then 0.3N KOH hydrolysis, 37°C 18 h	12
5. Heat denaturation, treatment with deoxyribonuclease 20 $\mu\text{g}/\text{ml}$ , 37°C 30 min and then 0.3N KOH hydrolysis, 37°C 18 h.	14

identical to that for dsRNA and the final fractions were pooled and concentrated. Table 3-3 summarises the results on the effect of alkaline hydrolysis of the material thus obtained. The table indicates that treatment of radioactive material (3,500 cpm) with 0.3 or 0.5N KOH, resulted in almost complete digestion. Very little radioactivity also remained after alkaline hydrolysis of material treated with heat denaturation, followed by deoxyribonuclease. The results indicate the third peak material is solely RNA and not a DNA-RNA hybrid.

### 3-4 DISCUSSION

#### 3-4-1 *Heat Denaturation Profile*

The purified fraction of RNA isolated from cellulose chromatography has been substantially confirmed as a double stranded molecule by the experiments in this chapter. Except for a small contamination with poly(A) segments, which will be discussed in greater detail in Chapter VI, it can be said that the third peak fraction can be taken as dsRNA. The experiments in turn by confirming the double stranded character of the purified material, strengthen the validity of the cellulose technique as a means of producing purified dsRNA.

One of the important tests for demonstrating the double stranded nature depends on the fact that dsRNA is stable and insensitive to ribonuclease at high salt concentrations, but unstable and sensitive at low salt concentrations. The dsRNA purified by cellulose column chromatography possessed this differential sensitivity to ribonuclease digestion and further

showed the typical transition curve upon heat denaturation. The transition (or denaturation) is accompanied by an abrupt shift in optical density at 260 nm, or in the case of dsRNA, an abrupt shift from ribonuclease resistant to ribonuclease sensitivity (GEIDUSECHEK *et al.*, 1972). The temperature at which this abrupt shift or transition takes place is the  $T_m$ . The  $T_m$  depends upon the ionic conditions during heat denaturation. In this case the measured  $T_m$  value shifts from 74°C to 94°C when the ionic conditions are changed from low to high salt. This denaturation temperature and biphasic curve are the most indicative property which describe dsRNA and consequently should be used as the first marker for double stranded character.

Another observation can be made from the biphasic curve, and that is the steepness of the curve at the point of inflexion. It is not possible to draw an exact parallel with the situation in DNA, but the behaviour of DNA sequences under thermal denaturation conditions is reviewed by BRITTEN and KOHNE (1968). The behaviour of dsRNA is similar to that of DNA - except that the  $T_m$  is generally higher in dsRNA than DNA with the equivalent nucleotide composition, secondary structure and molecular weight (BILLETER *et al.*, 1966; BISHOP and KOCH 1967; GOMATOS and TAMM 1963). If we parallel the situation described for DNA strands (BRITTEN and KOHNE 1968), we expect that the broader range of dissociation the more likely is the imprecise pairing, *viz* the more unlikely

(except for the poly(A) content, as in the case of dsRNA)



that exactly paired regions along the entire length of the RNA-RNA strand exist. ~~Another implication may, however, be that the double stranded molecules may have single stranded ends. Between these ends there may be some pairing and mismatching, which would also account for broadness in the transition region.~~

Without further careful measurements it is not possible to predict the exact amount of mismatching between the two strands and whether or not any of this is due to mismatching between paired single stranded end regions of dsRNA. Nevertheless, it appears that some mismatching of bases does occur.

#### 3-4-2 *Base Analysis*

The base analysis values reported for ribonuclease resistant RNA differ markedly in the literature (STERN and FRIEDMAN 1970, 1971; HAREL and MONTAGNIER 1972). Different techniques of preparation and purification may account for these differences. In many cases, the lack of purification may account for erroneous estimations. The values most comparable to dsRNA from rat liver are those of estimations obtained on sea urchin dsRNA (KRONENBERG and HUMPHREYS 1972). Although none of the individual mononucleotide values are the same, as would be expected, since the sources, purification methods and tissues are completely different; there is some similarity in the high adenylic acid value obtained. No comment, however, was made on this high reported value.

The values given here are those of purified dsRNA (except for the poly(A) contamination, as mentioned) and in

this case the  $A/U$  and  $G/C$  ratios are both nearly equal to 1. This is strong evidence to support a base paired structure.

### 3-4-3 Relationship of $T_m$ and $G + C$ Content

It is important to comment here on the agreement between the value of  $T_m$  obtained by heat denaturation and that predicted from the  $G + C$  content obtained by base analysis.

A linear relationship between  $G + C$  content and  $T_m$  values for dsRNA was found by VAN GRIENSVEN *et al.* (1973). The values of  $G + C$  content and  $T_m$  by hyperchromicity or ribonuclease resistance measurements, was obtained from the studies of various workers using viral dsRNA (for references see Table 3-2). If the rat liver  $G + C$  content is plotted on this relationship, the expected  $T_m$  value is only  $1^\circ\text{C}$  different from that actual measurement obtained under high salt ionic conditions. This result shows that a linear correlation can be applied to the properties of a non-viral dsRNA and provides additional evidence for the values obtained for either rat liver dsRNA content or  $T_m$  measured values.

There has been no systematic comparison of  $T_m$  and base composition values of dsRNA purified from uninfected cells. This study presents the first expression of such a correlation with ~~uninfected~~ <sup>from uninfected cells</sup> dsRNA, KRONENBERG and HUMPHREYS (1972), who prepared a sea urchin embryo dsRNA fraction by similar means, reported a  $T_m$  of  $76^\circ\text{C}$  in low salt, but unfortunately made no measurement at high salt concentrations. From their published  $G + C$  content (.41) we would expect a value of  $94^\circ\text{C}$  for

the  $T_m$  under high salt conditions, using the relationship discussed above.

#### 3-4-4 *Caesium Sulphate Density Gradient Fractionation*

This method of analysis is dependent upon the property that the buoyant density of double stranded polynucleotides is lower than that of single stranded molecules in concentrated salt conditions, (SUEOKA, MARMUR and DOTY 1959) a fact generally attributed to the greater hydration of the double stranded helix. The use of caesium sulphate as against caesium chloride for gradients is necessary because of the elevated temperatures which would be required to prepare sufficiently dense solutions of caesium chloride due to the high buoyant density of RNA preparations. The sedimentation pattern of rat liver dsRNA does indicate a peak at a lower buoyant density than that observed for single stranded RNA, but not as low as the observed value for DNA. This is in accord with the expected behaviour as a dsRNA molecule. ~~In addition, the double stranded behaviour is confirmed by the independent observation of a similar value for the buoyant density of a well known dsRNA molecule from reovirus. This analysis constitutes independent evidence for the double stranded nature of the isolated fraction.~~

Caesium sulphate density gradient analysis has been explored by two other research groups. KIMBALL and DUESBERG 1971, used this method to correlate peaks of ribonuclease resistant material with viral titre reduction patterns, and hence interferon production associated with dsRNA. They also



reported the peaks of ribonuclease resistant RNA lying between reovirus dsRNA were used to confirm the reaction of the DNA and single stranded viral RNA on caesium sulphate density gradient analysis. More recently, JELINEK and DARNELL (1972) used this behaviour on caesium sulphate gradient analysis as the sole identifying feature of the ribonuclease resistant RNA isolated from Hela cell nuclei. They reported the peak on the gradient as being identical in value to a reovirus dsRNA similarly analyzed.

#### 3-4-5 *Immunodiffusion*

The reaction of this dsRNA preparation to specific antigens provides independent evidence of double stranded character. This technique had been used specifically to detect dsRNA from infected sources (SCHWARTZ and STOLLAR 1969; STOLLAR and STOLLAR 1970; SCHUR and MONROE 1969). More recently, FRANCKI and JACKSON (1972) used this technique to investigate ~~another infectious~~ <sup>from virus-infected organisms.</sup> dsRNA. Using this immunodiffusion technique, it was possible to detect the dsRNA from an uninfected source, namely rat liver dsRNA. In a later chapter the detection of dsRNA from rat testes will be described, but the method employed was identical. The anti-serum is specific for a synthetic double stranded RNA molecule, poly(I).poly(C), but also reacts to natural dsRNA's. It does not, however, react at all to either double stranded DNA or single stranded RNA. Consequently the heat denaturation of the natural rat liver dsRNA results in single strands, which then do not react to the antiserum. Poly(I).poly(C) and

reovirus dsRNA were used to confirm the reaction of the antiserum to dsRNA's. This method of detecting dsRNA is particularly useful where unlabelled, or difficult to label, dsRNA's are suspected, but where the quantity of material is relatively small.

### 3-4-6 Size

The first sections of the discussion have dealt with confirmatory evidence for dsRNA as well as providing some detail of the properties of that double stranded molecule. The question of size, however, deals more specifically with a particular property of rat liver dsRNA.

The experiments demonstrated that dsRNA purified from ribonuclease resistant rat liver whole cell RNA was composed of material electrophoresing as two distinct peaks. The first peak was heterogeneous and migrated more slowly than the marker tRNA. The second was homogeneous in size and had a mobility which is slightly greater than that of the tRNA. This would indicate the existence of two distinct species of dsRNA, a possibility which was hinted at by the G-200 Sephadex separation of ribonuclease resistant RNA. The two species, however, have distinctly different characteristics; the slower moving species, which was high in specific activity, indicates a range of different sized dsRNA's. The fast moving species, low in specific activity, was very homogeneous, indicating probably a single species of dsRNA of uniform size. Most recently, RYSKOV *et al.* (1973), reports the presence of

two separate species of dsRNA in a ribonuclease resistant RNA preparation. Some doubt remains as to whether the two peaks observed in our experiments exactly correspond to those obtained in their preparation. In particular, their preparation of short dsRNA species is characteristically G + C rich in base composition and very heterogeneous in size. A more detailed characterization is required for precise comparison of these species.

It is difficult to estimate with accuracy the exact size of the dsRNA's from the result obtained on gels. tRNA, which was used as a marker on the gels and which has a sedimentation constant of 4S, has a polynucleotide chain of about 80 nucleotides and a molecular weight of 25,000. In the gel pattern observed, the two species of dsRNA run slightly slower and slightly faster than the tRNA. It is tempting to therefore indicate that the dsRNA species are in 2-3S and 5-6S positions respectively, however this is based on the assumption that tRNA and dsRNA have the same mobility on 10% acrylamide gels. HARLEY, WHITE and REES (1973) carefully compared the mobilities of double and single stranded RNA's at varying gel concentrations. They report major differences in the electrophoretic mobility of both double and single stranded RNA's and DNA at the same gel concentration. Unfortunately tRNA was not analyzed for its mobility with respect to either of these species. The differences in mobility between double and single stranded species depends upon a number of factors. It is apparent that the length of the double stranded RNA is also important in determining the



mobility. It also appears that high molecular weight dsRNA behaves like DNA in showing a limiting lower mobility value at any fixed gel concentration, *viz* it runs faster than we would expect a single stranded RNA of the same molecular weight would run. Whether or not low molecular weight dsRNA behaves similarly is not clear, but it seems that the secondary and tertiary structure of RNA and DNA are important for the mobility of the species in gels. There is a suggestion that double stranded linear DNA molecules are aligned parallel to the direction of travel in polyacrylamide gel (FISHER and DINGMAN 1971; AAIJ and BORST 1972; LOENING 1969; HARLEY, WHITE and REES 1973), and hence have increased mobility; the clover leaf structure for tRNA was proposed by HOLLEY *et al.*, (1965). In this structure, 44% of the bases are found in double stranded regions. These regions, however, are not longer than 6-7 nucleotide pairs. The conformation of rat liver dsRNA is unknown, but it may be that tRNA and rat liver low molecular weight dsRNA are not very dissimilar in their behaviour on gel electrophoretograms.

The observation that purified dsRNA sediments in the 4-5S region on a sucrose density gradient is support for the conclusion reached on the size of the dsRNA molecule. In any case, the estimated size of the dsRNA does not appear to be greater than 7S. Ribonuclease resistant RNA, although not purified, gave an indication of a peak in the 4-5S region on G-50 Sephadex. It is therefore clear that tRNA

is not an unreasonable marker for estimation of molecular weight of dsRNA on gels. Although there is uncertainty of the behaviour of dsRNA under gel conditions, <sup>if it is assumed that tRNA has a secondary structure and electrophoretic mobility</sup> the results <sup>not unlike dsRNA</sup> obtained give an approximate estimation of 80-120 nucleotides for the broadly dispersed dsRNA species.

The smaller dsRNA may be as small as 50 nucleotides, but may be larger, depending on whether its conformation is responsible for increased mobility when compared with tRNA.

### 3-4-7 DNA-RNA Hybrid

The evidence of potassium hydroxide hydrolysis confirms that dsRNA is not wholly or partly a DNA-RNA hybrid. Rat liver DNA is not synthesized under the ordinary labelling conditions of RNA (1 hour label), however, because of the small fraction of purified dsRNA being isolated; some of the material may have been due to a DNA-RNA hybrid. Hence the DNA was labelled in rat liver by using a long labelling period and in regenerating rat liver, where active DNA synthesis is known to occur. The ribonuclease resistant fraction was isolated and chromatographed normally, and the resulting labelled third peak species tested for DNA by hydrolyzing the labelled species. No label remained after potassium hydroxide hydrolysis. If any DNA had been present, and hence a DNA-RNA hybrid, radioactivity would have remained. All but a very few counts remained.

STERN and FRIEDMAN (1970, 1971) were the only other workers to mention a DNA-RNA hybrid. They used a [ $^3\text{H}$ ]-thymidine label in Burkitt's lymphoma cells, and isolated

ribonuclease resistant RNA, synthesized in the presence of actinomycin D. They also found no thymidine label incorporated into their RNA.

The discussion in this chapter has brought to light various properties of rat liver dsRNA, as well as substantiating the isolation of a double stranded species. The investigations were conducted over a broad range of properties particularly applicable to double stranded RNA species. There are some indications from the two sizes and different specific activities that at least two distinct types of dsRNA rat liver may exist. The following chapters will deal with the distribution within the cell of dsRNA, and the properties of those dsRNA species isolated. Other properties of interest, such as the coexistence with poly(A), will be discussed.

In this chapter several techniques are used to fractionate the cell into its components, nucleus and cytoplasm, and investigations of these components conducted to obtain clues as to the origin. The technique of fractionation is particularly important. Other workers are not always able to fractionate the cell components successfully, i.e. tissue cultured cells are very difficult to divide into nucleus and cytoplasm. The cytoplasm adheres to the nuclei, and techniques using detergents are generally unsatisfactory (Mann, 1970). Consequently measurements of nuclear RNA species can be frustrated by cytoplasmic contamination. The choice of rat liver as the tissue of investigation excludes these difficulties because reliable methods of fractionation



## CHAPTER IV - BIOLOGICAL PROPERTIES OF dsRNA - CELLULAR AND TISSUE DISTRIBUTION

### 4-1 INTRODUCTION

It is particularly important in investigating the biological properties of dsRNA, to establish the origin. The origin of dsRNA gives definite implications of the particular function(s). The question of origin is divided into two parts, (1) Where is dsRNA located in the cell? (2) What type of molecular species is dsRNA derived from or does it exist as an independent molecule? These two questions will be dealt with in separate chapters. The cellular origin will be investigated in this chapter and the molecular origin in the next.

In this chapter several techniques are used to fractionate the cell into its components, nucleus and cytoplasm, and investigations of these components conducted to obtain clues as to the origin. The technique of fractionation is particularly important. Other workers are not always able to fractionate the cell components successfully, (e.g. tissue cultured cells are very difficult to divide into nucleus and cytoplasm. The cytoplasm adheres to the nuclei, and techniques using detergents are generally unsatisfactory, (MONCKTON 1970). Consequently measurements of nuclear RNA species can be frustrated by cytoplasmic contamination. The choice of rat liver as the tissue of investigation excludes these difficulties because reliable techniques of fractionation

(i.e. the clean preparation of nuclei) are well established, allowing absolute decisions to be taken about dsRNA origin, where only nuclear species have been isolated.

Other important experiments in this section include the investigation of synthesis of dsRNA in the presence of actinomycin D, under conditions which are known to prevent rRNA or precursor rRNA synthesis. Similarly the time for incorporation of radioactive RNA precursors is investigated to give indications of the metabolic turnover of dsRNA.

The components are fractionated in each case and the individual whole RNA extracted *viz* cytoplasmic RNAs and nuclear RNAs. The sedimentation analysis of these RNAs is reproduced, for although ultimately the RNA was ribonuclease digested, it is important to establish that intact undegraded species of RNA can be produced by the techniques outlined. If degradation with ribonucleases occurred during the preparation of intact cellular or nuclear RNA species, double stranded regions may be released from the intact species making it difficult to identify as an individual species or as part of another molecule of higher molecular weight.

The ribonuclease digestions undertaken to prepare dsRNA from separate cytoplasmic and nuclear RNA species are undertaken in an identical manner to that used for whole cellular RNA. The ribonuclease resistant RNA prepared is examined on cellulose chromatography to allow identification of the presence or absence of dsRNA. The separation method and the significance of this analysis method to prepare dsRNA,

has been discussed at length in Chapter II.

Taken overall, specific digestions of the individual extracted RNAs from specific cellular fractions, together with the studies of metabolic turnover and the synthesis of RNA species in the presence of actinomycin D, enable the pinpointing of the sources(s) of dsRNA within the cell.

The second aim of the investigation conducted in this chapter was the examination of other tissues for dsRNA. This is important for two reasons. The first is to establish the universality of the existence of dsRNA, and hence to suggest that the dsRNA is not particularly confined to the liver or associated with a liver-specific function, but is rather a new species of RNA which has a definite role in the normal metabolism and function. The second is to add weight to the evidence that dsRNA is not of viral origin.

#### 4-2 MATERIALS and METHODS

All materials and methods are identical to Chapters II and III, only additional methods are described below.

##### 4-2-1 *Materials*

Actinomycin D was purchased from Calbiochem.

##### METHODS

##### 4-2-2 *Labelling*

Isotope injections were identical to the procedure described in Chapter II. The time for incorporation of radioactively labelled precursor was 60 min, except where the incorporation time was 20 min.

##### 4-2-3 *Preparation of Nuclei*

Nuclei, free of all cytoplasm, were <sup>prepared</sup>~~mapped~~ according to the established methods previously described (CHAVEAU,



staining with gentian violet. Electron and optical micro-

MOULE and ROUILLER 1956; AMANO 1967; NAORA and KODAIRA 1969). This method, with slight modifications, is described below.

AMANO, Nuclei were prepared from rat liver which had been labelled as described. Livers were homogenized in 10 volumes of 0.25M sucrose containing 3.3 mM  $\text{CaCl}_2$ . Homogenization was carried out in a Waring blender at 50V for 2 min at 0°C. The homogenate was then centrifuged at 3750 (2,250 x g) rpm for 10 min in the GSA rotor of the RC2B Sorvall centrifuge. The supernatant was carefully removed and discarded. The precipitate was resuspended in 2.4M sucrose, 3.3 mM  $\text{CaCl}_2$ . The amount of 2.4M sucrose solution added was calculated as approximately 90 mls of solution per original liver (liver weights averaged 5 g consistently with 155-160 g rats). A further homogenization at 50V for 1 min was conducted. This homogenate was then centrifuged in sealed Beckman tubes at 21,000 rpm (44,000 x g) for 50 min in a Beckman type 21 preparative rotor. The nuclei are clearly separated by this process as they deposit on one side of the tube and the cytoplasmic debris on the other in dense sucrose solution. The cytoplasm was removed and the tubes thoroughly cleaned to remove residual cytoplasm. The nuclei from each tube were carefully suspended in 20 ml of 0.88M sucrose, centrifuged at 4000 rpm (1900 x g) in the SS-34 rotor of the RC2B Sorvall centrifuge, and the pelleted nuclei finally resuspended in 15 ml of 0.25M sucrose, containing 25  $\mu\text{g/ml}$  of potassium polyvinyl sulphate. These represent clean nuclei, and were routinely examined after

staining with gentian violet. Electron and optical microscopic examination revealed that nuclei are free of all cytoplasm. The reliability of this procedure has been adequately described (CHAVEAU, MOULE and ROUILLER, 1956; AMANO, 1967; NAORA and KODAIRA, 1969; NAORA, 1973).

#### 4-2-4 *Extraction of Nuclear RNA*

The nuclei were extracted by a procedure identical to that described for whole cell RNA (section 2-2-6). Protease digestions were omitted in the purification of nuclear RNA in some experiments.

#### 4-2-5 *Preparation of Cytoplasm*

Cytoplasm was prepared from labelled livers in 0.25M sucrose solution, but  $\text{Ca}^{++}$  was replaced by  $\text{Mg}^{++}$  in a similar manner to that previously described (NAORA and NAORA, 1967).

Homogenization was carried out, using a similar technique to that described for nuclei and whole cell homogenates, except that homogenization was very gentle, using a Waring blender, 30V for 1 min and 40V for 1 min. The homogenate was centrifuged at 13,000 rpm (20,000 x g) in the SS-34 rotor of the RC2B Sorvall centrifuge to remove all nuclei and whole cell debris. Microscopic examination showed that the supernatant was almost completely free of nuclear material.

#### 4-2-6 *Extraction of Cytoplasmic RNA*

The procedure for extraction of RNA from the cytoplasmic fraction prepared was identical to that previously described for whole cell homogenates.

#### 4-2-7 *Preparation of dsRNA from Cytoplasmic and nuclear RNAs*

The ribonuclease resistant RNA was prepared from purified cytoplasmic RNA and nuclear RNAs by treatment with ribonucleases A and  $T_1$  under conditions similar to that described for whole cell RNA. dsRNA was prepared from the same ribonuclease resistant RNA as has already been described, by cellulose chromatography and pooling of the third peak (dsRNA) fraction.

#### 4-2-8 *Preparation of Rat Testes Cellular RNA*

Four or five rats were sacrificed and their testes removed. These were minced with scissors, and homogenized in 10 volumes of 0.25M sucrose and 1% bentonite solution, as has already been described for cellular RNA. For preparation and purification of cellular testes RNA, the same procedure as rat liver cellular RNA was adopted, except that the concentration of deoxyribonuclease was increased to 50  $\mu$ g/ml.

#### 4-2-9 *Removal of Deoxyribonuclease Digest from cellular RNA*

The cellular testes RNA was found to contain substantial amounts of oligo-deoxyribonucleotides resulting from deoxyribonuclease treatment. This contaminant was removed by sucrose density gradient centrifugation.

The purified cellular RNA was dissolved in 4 ml of 10 mM Tris-HCl, 0.14M NaCl buffer pH 7.4, (RNA concentration 530 OD<sub>260</sub> nm units/ml) and 0.5 ml layered on 10-40% sucrose gradients made in the same buffer. The gradients were preformed and after addition of the sample, were then centrifuged at



26,000 rpm (88,000 x g) in a Beckman SW27 swinging bucket rotor for 16 h at 4°C. The gradients were fractionated using an ISCO density gradient fractionator equipped with a 2 mm optical cell. 1 ml fractions were collected as indicated, and the fractions (> 8S) pooled. Contaminating oligo-deoxyribonucleotide fragments were located almost at the top of the gradient. Two volumes of cold ethanol were added to the pooled fractions and precipitated at -20°C overnight. The precipitate was centrifuged on the bench centrifuge at 2,500 rpm. The pellet was washed and re-centrifuged twice with ice-cold 80% ethanol. The final precipitate was redissolved in 1 ml of 10 mM Tris-HCl, 1 mM MgCl<sub>2</sub>, 0.3M NaCl, pH 7.4. This represented a purified fraction of testes cellular RNA, free of DNA and deoxyribonuclease digestion products.

#### 4-2-10 *Preparation of Testes dsRNA*

The DNA-free cellular rat testes RNA was digested with ribonucleases A and T<sub>1</sub> in a similar manner to that described for rat liver cellular RNA (section 2-2-8). This ribonuclease resistant RNA was chromatographed on cellulose and the third peak fraction pooled and concentrated as previously described. This represented purified testes dsRNA.

#### 4-2-11 *Preparation of Actinomycin D Treated Cellular RNA from Rat Liver*

Rats were labelled intravenously for cellular RNA with isotope, as described previously (section 2-2-3).

Actinomycin D was administered in two separate intraperitoneal injections. A dose of 150  $\mu\text{g/kg}$  body weight was given in two equal parts, the first an hour before the intravenous injection of isotope, and the latter immediately after to ensure the effect of actinomycin D.

Preparation of actinomycin D treated cellular RNA and dsRNA was exactly as described for cellular RNA.

#### 4-2-12 *Sucrose Gradient Analysis of Nuclear and Cytoplasmic RNA's*

Sucrose gradient analysis was performed by dissolving RNA samples in 10 mM Tris-HCl, 0.14M NaCl pH 7.4 buffer. RNA (2.5  $\text{OD}_{260}^{\text{nm}}$  units/ml) was layered on to preformed 5-40% sucrose gradients made in the same buffer. The gradients contained a total volume of 4.8 ml and were centrifuged in an SW50L Beckman rotor at 39,000 rpm for 4 hrs, or 49,000 rpm for 3 hrs (130,000 or 200,000  $\times g$  respectively).

Gradients were fractionated using an ISCO density gradient fractionator with 10 mM light path cell, 3 drop fractions collected, and after the addition of 10 ml of Bray's scintillation cocktail or AQUASOL the radioactivity was counted.

### 4-3 RESULTS

#### 4-3-1 *Effect of Labelling Time on the Occurrence of Label in dsRNA*

The aim of this experiment was to discover whether dsRNA is labelled as rapidly as the HnRNA.

Rat liver was labelled intravenously for cellular RNA, as described previously, but the labelling time, viz, the time between administration of the isotope and sacrificing the animal and removing its liver, was reduced to 20 min. Within

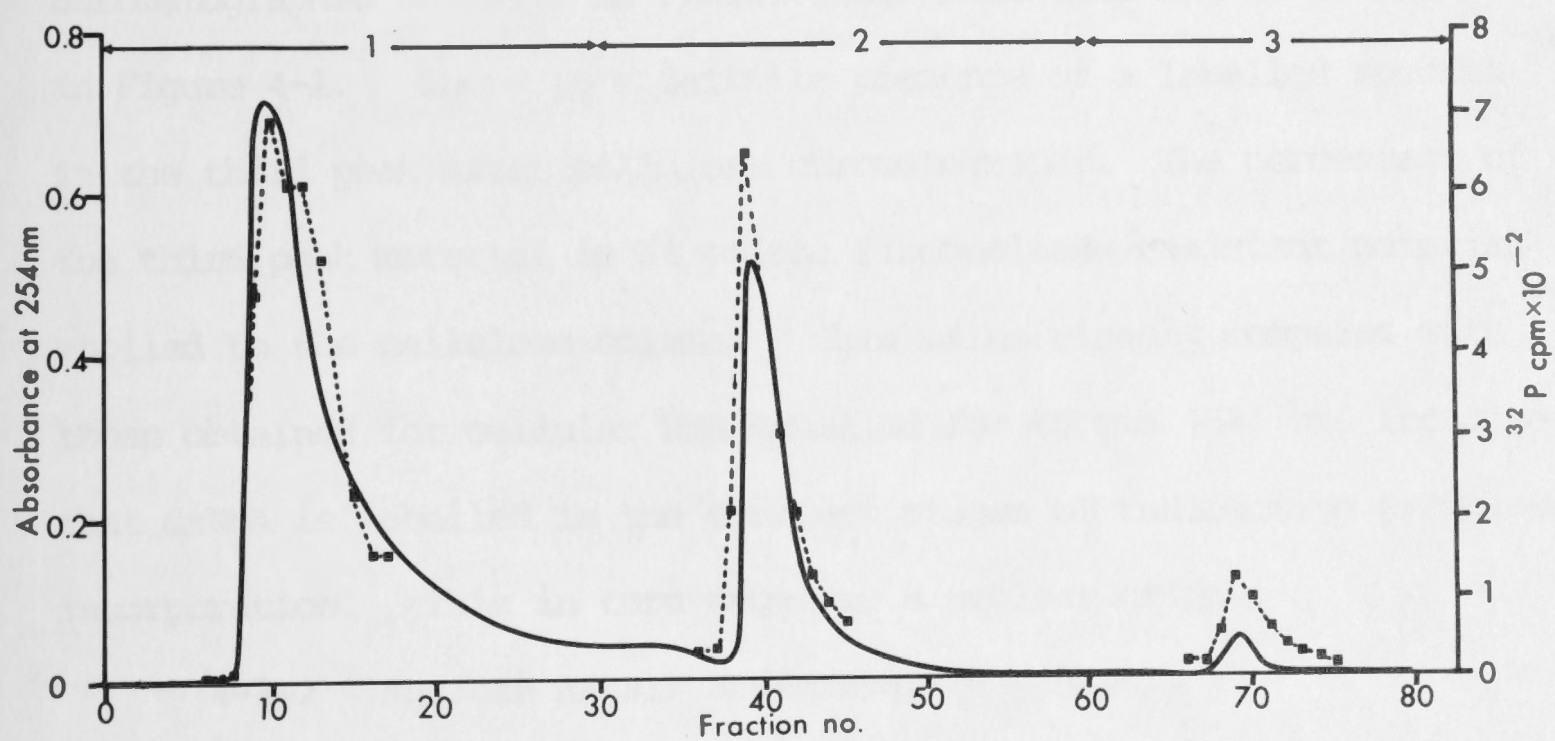
FIGURE 4-1 Cellulose column chromatography of ribonuclease  
resistant RNA isolated from whole rat liver  
cells labelled intravenously with  $^{32}\text{P}$  for  
20 min

Animals were sacrificed after a 20 min labelling period. The purified ribonuclease resistant RNA was dissolved in the first TSE:ethanol solution. 1, 2 and 3 represent elution with TSE:ethanol in the ratios of 65:35, 85:15 and TSE buffer alone, respectively. The RNA was applied to a (1.5 x 20 cm) cellulose column, and 3 ml fractions collected.

\_\_\_\_\_, Absorbance at 260 nm.

■.....■, Trichloroacetic acid precipitable  
radioactivity in 1 ml of each column  
fraction.





this short period it was reported that only nuclear RNAs except for the terminal addition <sup>to</sup> of tRNA, are labelled and these RNAs would not have migrated to the cytoplasm (MURAMATSU, *et al.* 1964; NAORA and KODAIRA, 1968), hence detection of radioactive label in the double stranded species hints at a nuclear origin.

The cellular RNA was isolated from these livers and the purified ribonuclease resistant RNA was chromatographed on cellulose. For this experiment <sup>32</sup>P-labelled livers were used and the result of cellulose chromatographic analysis of ribonuclease resistant RNA is illustrated in Figure 4-1. There is a definite presence of a labelled species in the third peak after cellulose chromatography. The percentage of the third peak material is 7% of the ribonuclease resistant material applied to the cellulose column. This value closely compares with those obtained for cellular RNA labelled for 60 min (6%) and indicates that dsRNA is labelled in the earliest stages of radioactive precursor incorporation. This in turn suggests a nuclear origin.

#### 4-3-2 Nuclear RNAs: Sedimentation Analysis

Nuclei, almost completely free of cytoplasm, were prepared from rat liver cells labelled with [<sup>3</sup>H] orotic acid for 60 min.

Analysis of the RNAs derived from extraction of these nuclei is shown in the sucrose density gradient profile illustrated in Figure 4-2. The optical density profile indicates the presence of 28S, 18S rRNA and 4S tRNA in the preparation of nRNA. The radioactivity profile however, shows a dispersion over the entire gradient with higher peaks in the 28S and 18S regions and a shoulder at 4S RNA, indicating that the nuclear RNAs consist of rRNA and pre-rRNA, tRNA and InRNA.

FIGURE 4-2    Sucrose density gradient profile of  $^3\text{H}$ -labelled  
rat liver nuclear RNA

Nuclear RNA was prepared from the livers of animals intravenously labelled with [ $^3\text{H}$ ] orotic acid for 1 h. The RNA was dissolved in 10 mM Tris-HCl, 0.14M NaCl, pH 7.4, and layered into preformed 5-40% sucrose gradients made in the same buffer. The gradient was fractionated into 3 drop fractions, and each fraction assayed for radioactivity after diluting in 1 ml of water.

\_\_\_\_\_ , Absorbance at 254 nm  
▲.....▲ , Radioactivity



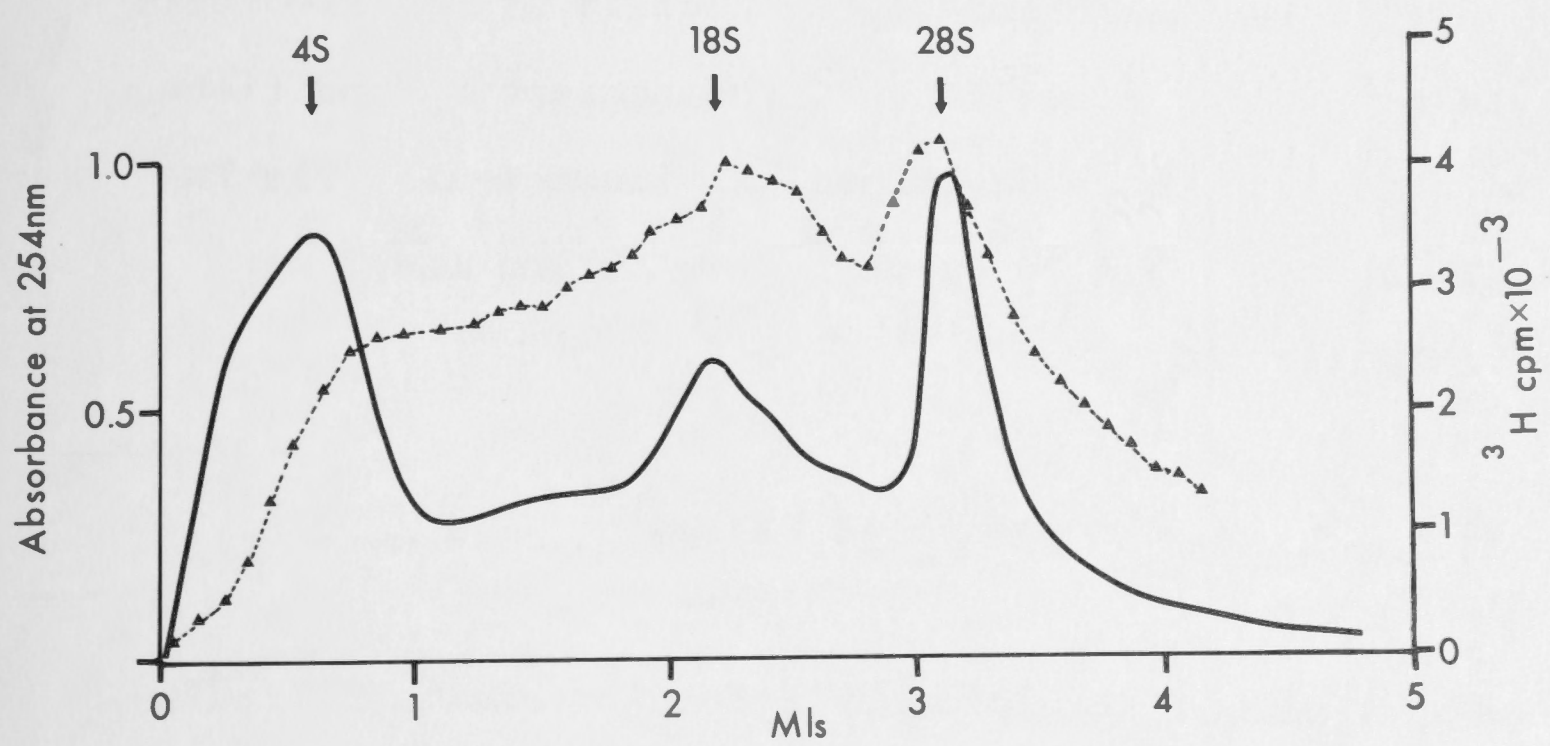
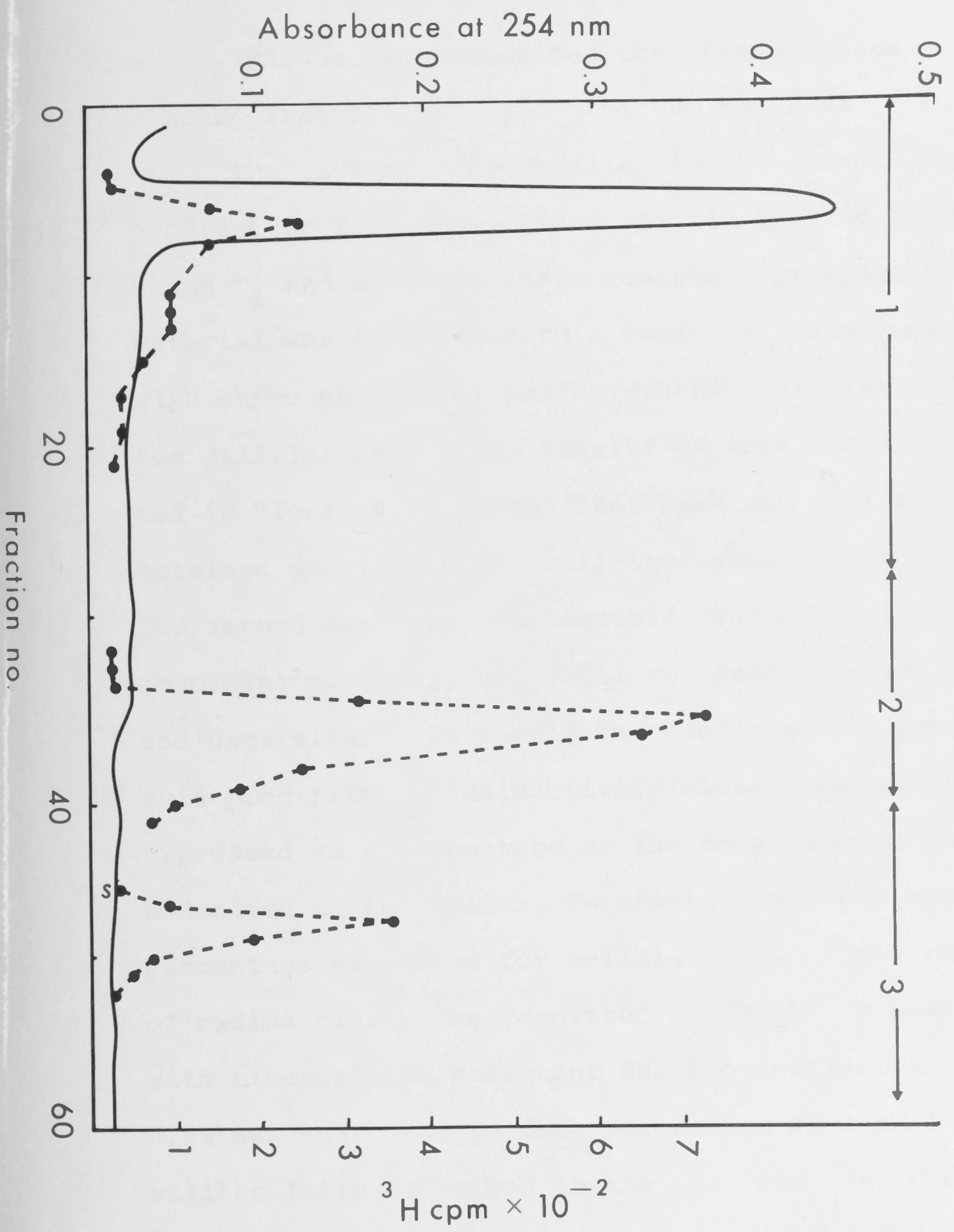


FIGURE 4-3    Cellulose column chromatography of  $^3\text{H}$ -labelled  
ribonuclease resistant RNA isolated from rat  
liver nuclei

Animals were labelled intravenously with [ $^3\text{H}$ ] orotic acid and sacrificed after 1 h. Chromatographic conditions were identical to those described in Figure 4-1. The RNA was applied to a 1.5 x 20 cm cellulose column and 3 ml fractions collected.

\_\_\_\_\_ , Absorbance at 254 nm

●.....● , Trichloroacetic acid precipitable radio-  
activity in 0.5 ml of each column fraction.





4-3-3    *Cellulose Chromatography Analysis of Purified  
Ribonuclease Resistant RNA Prepared from Nuclear  
RNAs*

This section describes the chromatographic analysis of a dsRNA fraction prepared from nuclear RNAs.

The nuclear RNAs detailed in the sucrose density gradient profile above, were further treated with ribonucleases A and T<sub>1</sub> and a ribonuclease resistant fraction prepared. This material was then added to a small cellulose column and eluted with three changes of buffer:ethanol, as previously described for cellular RNA. The results of this analysis are illustrated in Figure 4-3. The first peak was similar to that obtained previously for cellular ribonuclease resistant RNA. The second peak was considerably reduced in ultra-violet absorbing material, but still retained some acid precipitable radioactivity. It should be noted that the percentage of acid precipitable radioactivity eluted in the third peak, expressed as a percentage of the total ribonuclease resistant RNA added to the column, is greater than the corresponding percentage expressed for cellular RNA. Approximately 15% of radioactivity was recovered in the third peak fraction with ribonuclease resistant RNA from nuclear RNA, whereas 6.0% was the corresponding percentage with cellular RNA. As will be fully described in the next chapter, the material recovered in the third peak was found to be dsRNA. These findings would suggest that dsRNA may be rich or exclusively present in <sup>uc</sup>neulei.

FIGURE 4-4     Sucrose density gradient profile of  $^3\text{H}$ -labelled  
rat liver cytoplasmic RNA

Cytoplasmic RNA was prepared from the livers of animals intravenously labelled with [ $^3\text{H}$ ] orotic acid for 1 h. Sucrose gradient fractionation on the 5-40% gradient was identical to that described in Figure 4-2.

\_\_\_\_\_ , Absorbance at 254 nm

▲.....▲ , Radioactivity

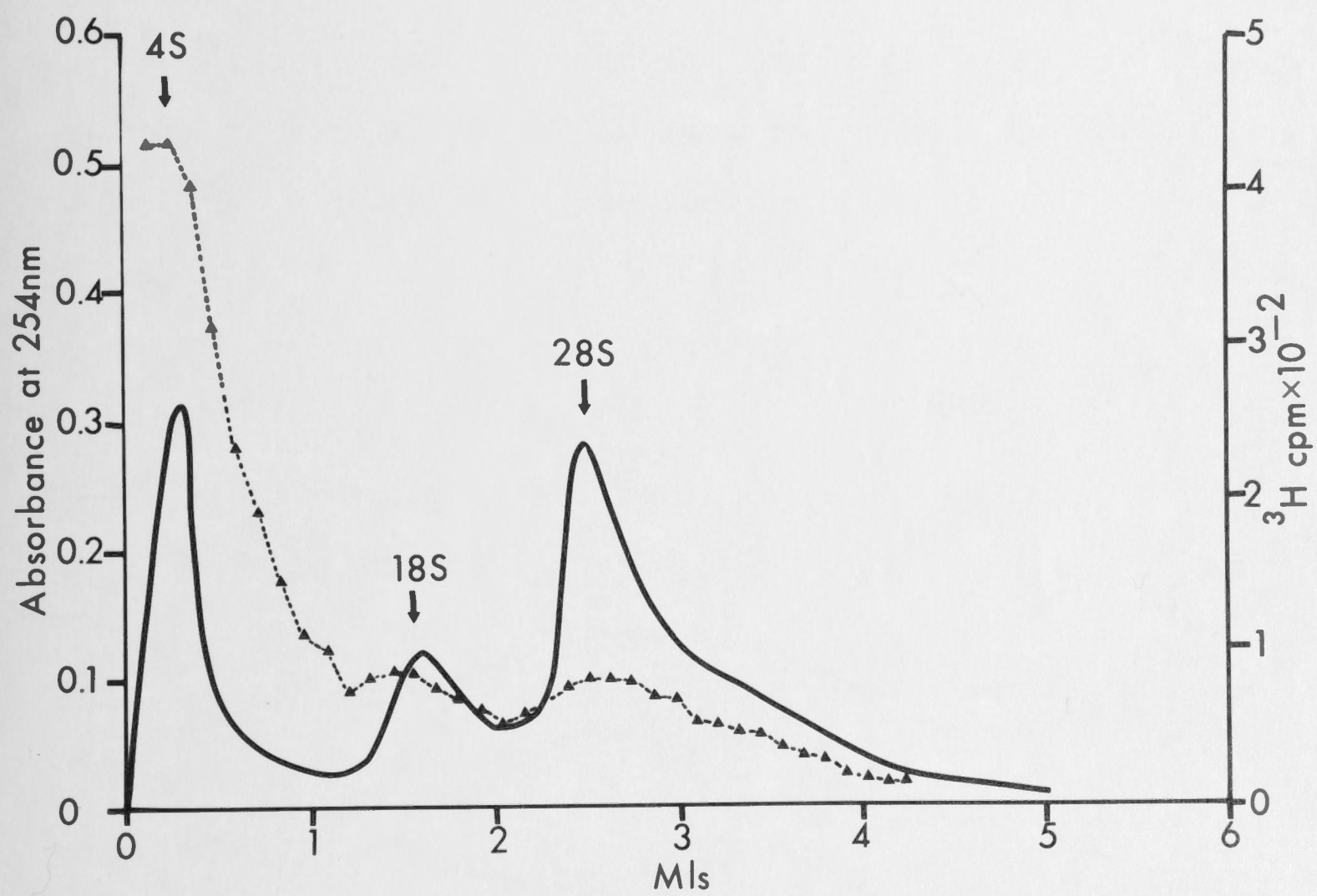


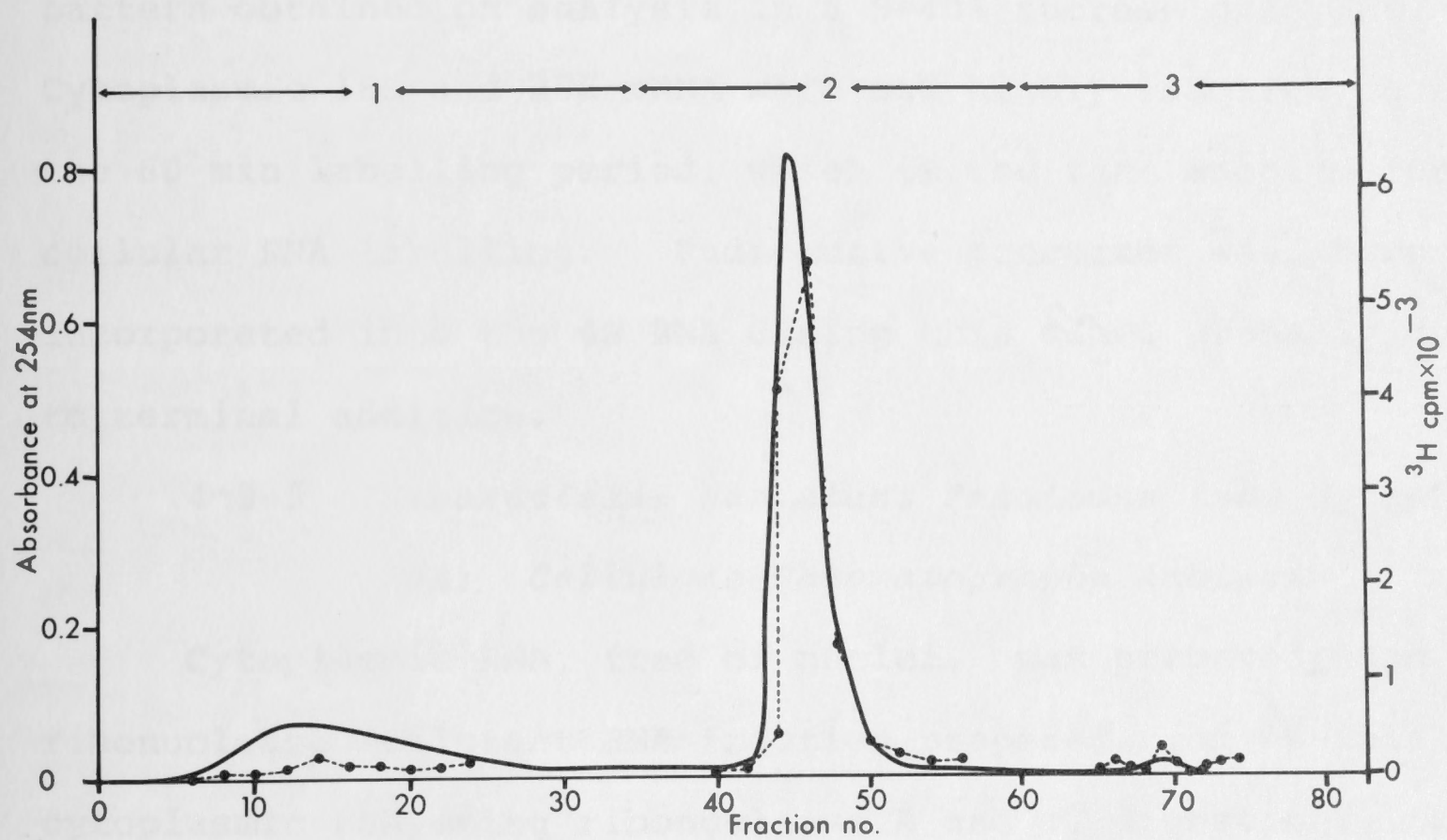


FIGURE 4-5    Cellulose column chromatography of  
<sup>3</sup>H-labelled ribonuclease resistant RNA  
isolated from rat liver cytoplasm

Animals were labelled intravenously with [<sup>3</sup>H] orotic acid and sacrificed after 1 h. Ribonuclease resistant RNA was isolated from cytoplasm prepared free of nuclei. Chromatographic conditions were identical to that described in Figure 4-1. 3 ml fractions were collected.

\_\_\_\_\_, Absorbance at 254 nm

●.....●, Trichloroacetic acid precipitable radioactivity in 1 ml aliquots of each fraction.



#### 4-3-4 *Cytoplasmic RNA: Sedimentation Analysis*

Cytoplasmic RNA was prepared from liver cells labelled with [ $^3\text{H}$ ] orotic acid for 60 min. The preparation of cytoplasm was found to be almost entirely free of nuclei, nuclear fragments and whole cells. Cytoplasmic RNA was prepared, extracted and purified in the same manner as described for cellular RNA. Figure 4-4 illustrates the sedimentation pattern obtained on analysis in a 5-40% sucrose gradient. Cytoplasmic 18S and 28S rRNA were not highly labelled during the 60 min labelling period, which is the time adopted for cellular RNA labelling. Radioactive precursor was, however, incorporated into the 4S RNA during this time, probably due to terminal addition.

#### 4-3-5 *Ribonuclease Resistant Fractions from Cytoplasmic RNA: Cellulose Chromatography Analysis*

Cytoplasmic RNA, free of nuclei, was prepared, and a ribonuclease resistant RNA fraction prepared from  $^3\text{H}$ -labelled cytoplasmic RNA using ribonuclease A and  $\text{T}_1$  digestions, as described for cellular dsRNA. This ribonuclease resistant fraction was analyzed for double stranded character by chromatography on cellulose.

Figure 4-5 illustrates the profile obtained. The material eluting in the third peak contained an extremely small proportion (0.3%) of acid precipitable radioactivity of the radioactivity associated with the ribonuclease resistant RNA applied to the column. The percentage is very low when compared to 6% obtained with cellular dsRNA and 15%



TABLE 4-1

Effect of Heat and Ribonuclease treatment on pooled 3rd peak fraction from cellulose chromatography of ribonuclease resistant RNA derived from cytoplasmic RNA.

<u>Treatment</u>	<u>cpm</u>
None	182
Heat denaturation only	123
Ribonuclease A digestion (20 $\mu$ g/ml, 30 min, 37°C)	12
Heat denaturation followed by Ribonuclease A digestion (20 $\mu$ g/ml, 30 min, 37°C)	14

with nuclear dsRNA fractions. Further examination of the third peak fraction revealed that this material did not behave as a double stranded structure. This was shown in the experiment in which the third peak fraction was pooled, concentrated and treated with ribonuclease before and after thermal denaturation. The result is illustrated in Table 4-1. A resistance to ribonuclease, normally lost after thermal denaturation, was not observed. This result clearly indicates that a majority or all of the rapidly labelled dsRNA found in cellular RNA does not exist in the cytoplasmic fraction and is of nuclear origin.

Detailed examination of cytoplasmic organelles, e.g. mitochondria or polysomes, was not conducted. Nevertheless, in Chapter VII the likelihood of a small dsRNA fraction within the mRNA will be discussed. The analysis of ribonuclease resistant RNA derived from the bulk of cytoplasmic RNAs may not be sufficiently refined to detect a very small proportion of dsRNA in mRNA or a small dsRNA associated with other cytoplasmic organelles.

4-3-6 *Cellular RNA prepared from Actinomycin D treated material: Isolation and Characterisation of a Double Stranded Fraction*

This section describes the evidence for a non-ribosomal origin for dsRNA which was obtained from the treatment of the animals with a low dose of actinomycin D prior to intravenous isotope injection. Animals were intraperitoneally injected with low doses of actinomycin D and intravenously

FIGURE 4-6     Sucrose density gradient profile of  $^3\text{H}$ -labelled  
rat liver cellular RNA isolated from the livers  
of animals treated with low levels of actinomycin  
D

Animals were treated with low levels of actinomycin D prior to, and immediately after, intravenously labelling with [ $^3\text{H}$ ] orotic acid to prevent rRNA and pre-rRNA synthesis. Sucrose gradient fractionation on 5-40% gradients was identical to that described in Figure 4-2. The position of the major RNA peaks obtained with an identical sample of  $^3\text{H}$ -labelled cellular RNA untreated with actinomycin D, are indicated.

▲.....▲ , Radioactivity of 3 drop fractions.



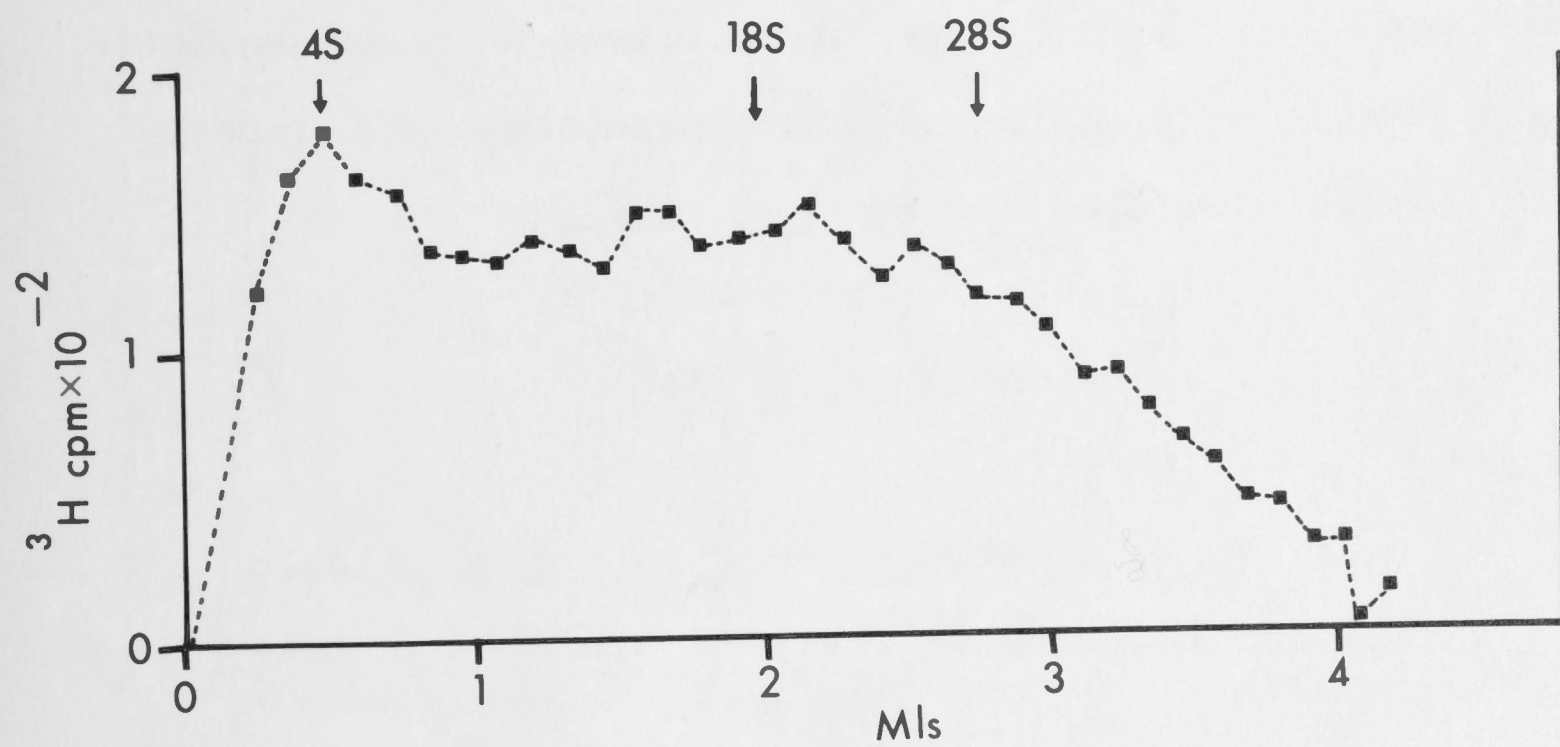
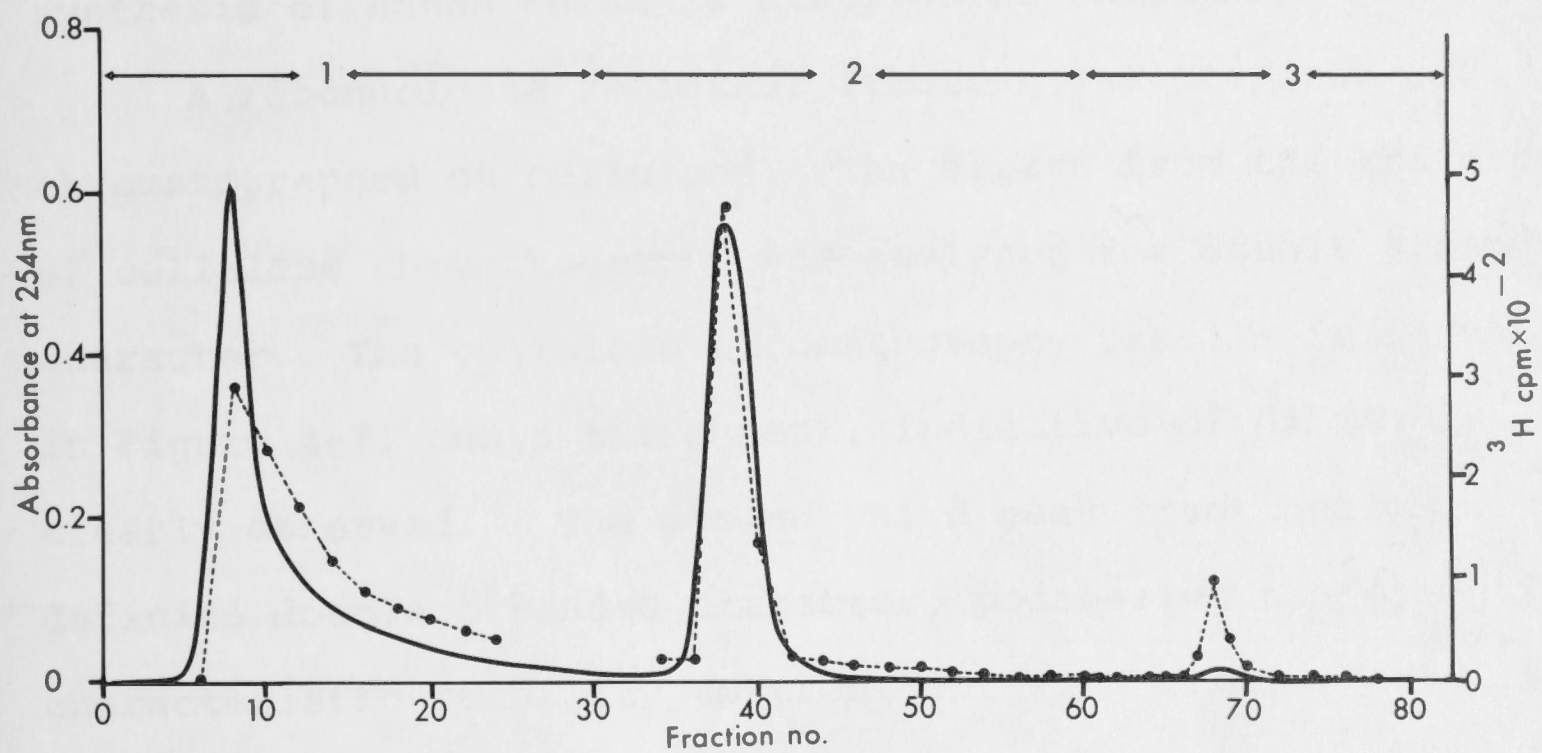


FIGURE 4-7     Cellulose column chromatography of  
<sup>3</sup>H-labelled ribonuclease resistant RNA isolated  
from the livers of animals treated with low  
levels of actinomycin D

Animals were treated with low levels of actinomycin D prior to and immediately after, intravenously labelling with [<sup>3</sup>H]orotic acid. Ribonuclease resistant RNA was prepared from whole cellular RNA from these livers. Chromatographic conditions were identical to those described in Figure 4-1. 3 ml fractions were collected.

\_\_\_\_\_ , Absorbance at 254 nm

●.....● , Trichloroacetic acid precipitable  
radioactivity in 0.5 ml aliquots  
of each fraction.





with [ $^3\text{H}$ ] orotic acid. Administration of the actinomycin D at a level of 150  $\mu\text{g/kg}$  body weight, resulted in a complete inhibition of all rRNA synthesis without appreciable effect on HnRNA synthesis (COZZONE and MARCHIS-MOUREN, 1967; NAORA and KODAIRA, 1967; WHITELAM and NAORA, unpublished observations). This was also seen in Figure 4-6, which illustrated the sucrose density centrifugation pattern of RNA's prepared from actinomycin D treated animals. Clearly there is no synthesis in the 18S and 28S regions, other than an overall synthesis of HnRNA which is distributed throughout the gradient.

A ribonuclease resistant fraction was prepared and chromatographed on cellulose. The eluate from the third peak of cellulose chromatography was analysed for double stranded character. The cellulose chromatography profile is illustrated in Figure 4-7, and a third peak, indicative of dsRNA, is clearly observed. The pooled third peak fractions show definite double stranded character, possessing the essential characteristic stability to treatment with ribonuclease, but losing this stability following thermal denaturation (Table 4-2). These observations provide evidence that dsRNA does not arise from rRNA or ribosomal precursor RNA and is probably from HnRNA.

#### 4-3-7 *Tissue Distribution of dsRNA Species: dsRNA in Purified Cellular RNA from Rat Testes*

Investigations were conducted to isolate a species of cellular RNA from rat testes and to determine whether dsRNA could be derived from this source. The existence of a dsRNA species in cells from other tissues would confirm that dsRNA

TABLE 4-2

Effect of heat and ribonuclease treatment on pooled third peak fraction from cellulose chromatography of ribonuclease resistant RNA derived from RNA synthesized in the presence of a low dose of actinomycin D.

<u>Treatment</u>	<u>cpm</u>
None	108
Heat denaturation only	117
Ribonuclease A digestion (20 $\mu$ g/ml 30 min, 37°C)	94
Heat denaturation followed by ribonuclease A digestion (20 $\mu$ g/ml 30 min, 37°C)	3

FIGURE 4-8     Sucrose density gradient profile of whole  
cellular RNA isolated from rat testes

Whole cellular RNA was prepared from rat testes and purified by extensive protease and deoxyribonuclease treatments. The RNA was dissolved in 10 mM Tris-HCl, 0.14M NaCl, pH 7.4 and layered onto preformed 10-40% sucrose gradients made in the same buffer. After centrifugation, the gradient was measured for absorbance and fractionated into 1 ml fractions. 4S + DNA indicates the 4S RNA peak with excess deoxyribonucleotide fragments. Fractions from 8 ml onwards were pooled for further analysis.

\_\_\_\_\_, Absorbance at 254 nm.



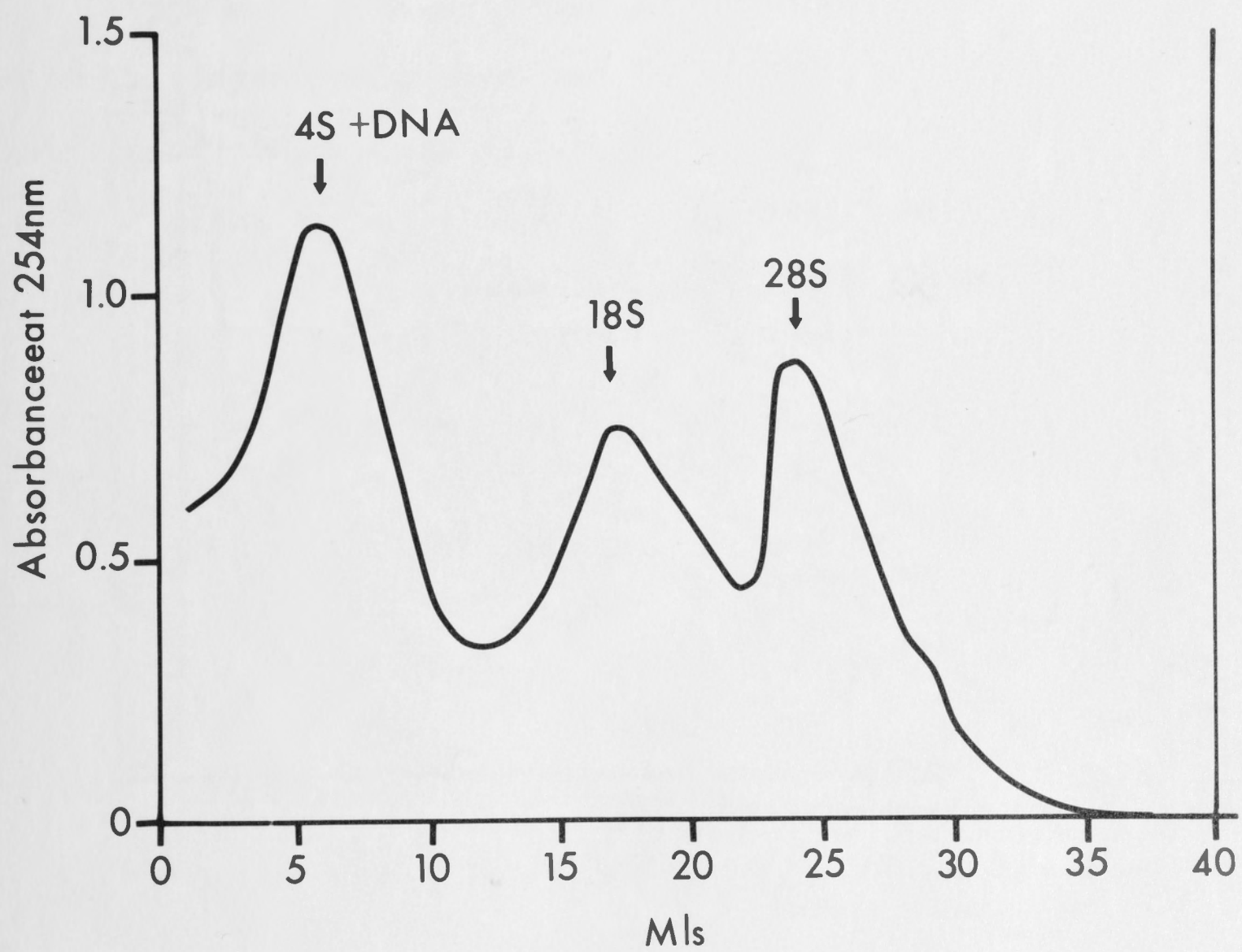
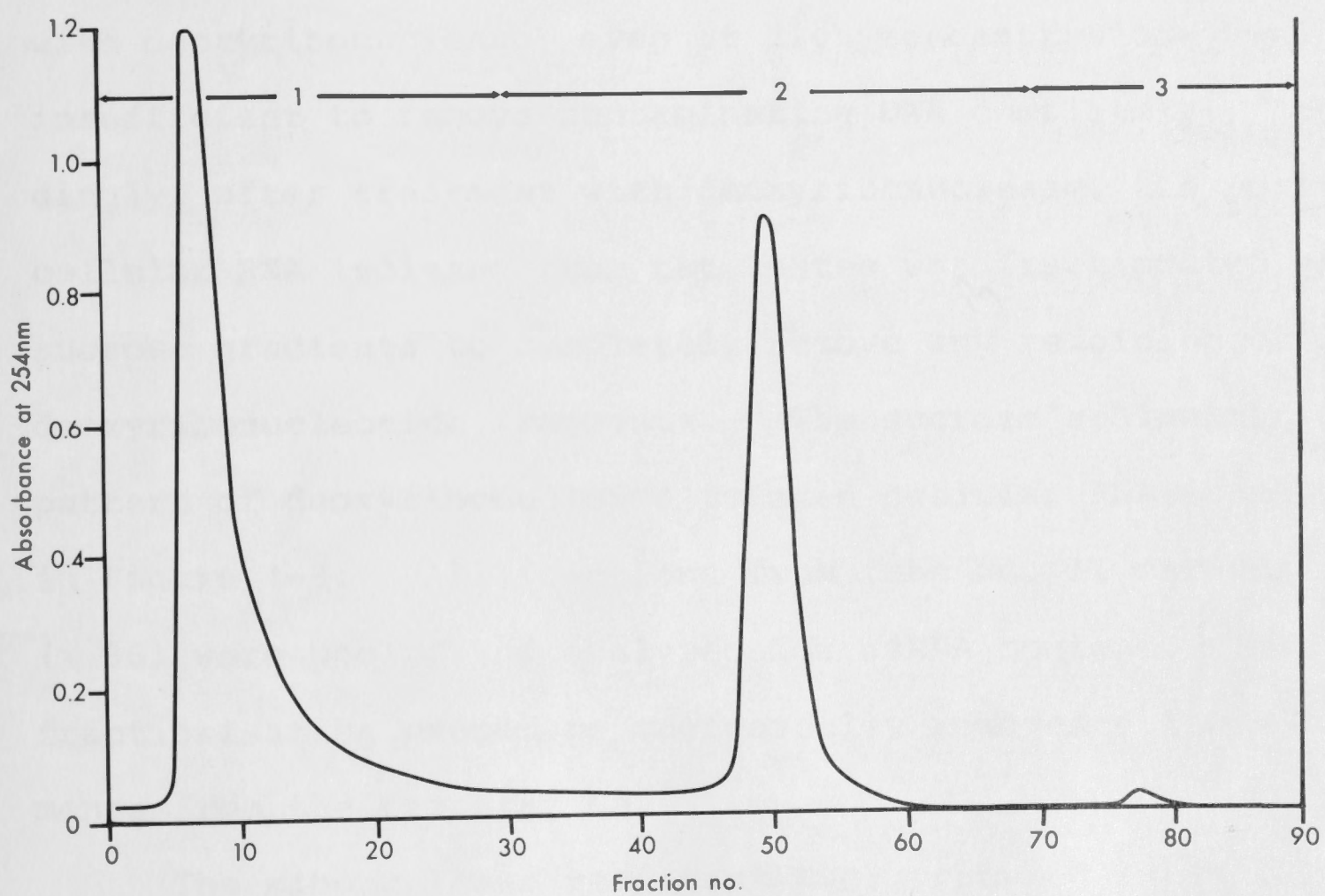


FIGURE 4-9    Cellulose column chromatography of ribonuclease  
resistant RNA isolated from purified whole  
cellular RNA of rat testes

Ribonuclease resistant RNA was prepared from whole cellular RNA pooled from the higher molecular weight fractions illustrated in Figure 4-8. Chromatographic conditions were identical to those described in Figure 4-1. A small cellulose column (1.5 x 20 cm) was used.

\_\_\_\_\_, Absorbance at 254 nm.





is present in cells universally, and is not exclusively present in liver or associated with a liver-specific function.

Extractions of cellular RNA from rat testes were conducted in a similar manner to that employed for rat liver cellular RNA, and the purification procedures were identical. Because of a large proportion of DNA in the testis relative to the RNA content, the RNA preparation was contaminated with substantial amounts of DNA. Consequently, simple digestion with deoxyribonuclease, even at high concentrations, was insufficient to remove contaminating DNA completely. Accordingly, after treatment with deoxyribonuclease, the purified cellular RNA isolated from rat testes was fractionated on sucrose gradients to completely remove any remaining oligodeoxyribonucleotide fragments. The sucrose sedimentation pattern of deoxyribonuclease treated cellular RNA is shown in Figure 4-8. All fractions from tube No. 11 onwards ( $> 8S$ ) were pooled and analysed for dsRNA content. This fractionisation procedure successfully removes all DNA fragments from the preparation.

The ribonuclease resistant RNA, prepared in an identical manner to that described for rat liver cellular RNA, is chromatographed similarly on cellulose. The  $OD_{254\text{ nm}}$  profile is illustrated in Figure 4-9. There is a definite indication of a third peak, eluting with TSE buffer. This would indicate the presence of a dsRNA fraction, since the behaviour on cellulose is identical to that described for rat liver cellular dsRNA.

Attempts were made to label the ribonuclease resistant fraction and dsRNA contained in the third peak. Two methods of isotopic labelling were employed; direct injection of [<sup>3</sup>H] orotic acid into the testes, and intravenous administration. Neither method produced a significant labelling of the ribonuclease resistant RNA. Consequently radioactivity in the third peak on cellulose chromatography was very low, and was insufficient to allow heat denaturation enzyme susceptibility analysis. An alternative confirmation of the double stranded nature of the third peak material was investigated,

4-3-8 *Confirmation of the Presence of dsRNA in Rat Testes*

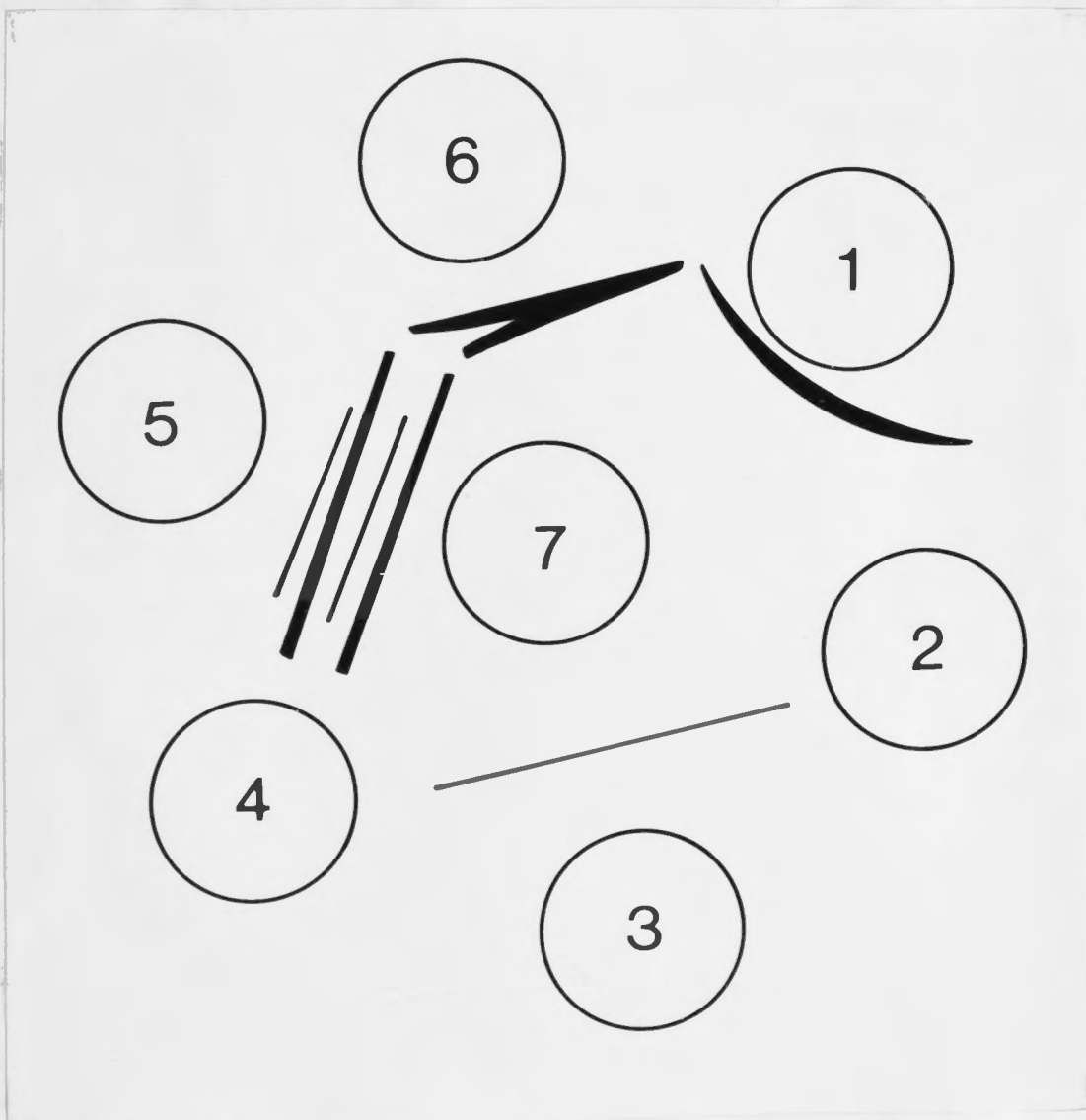
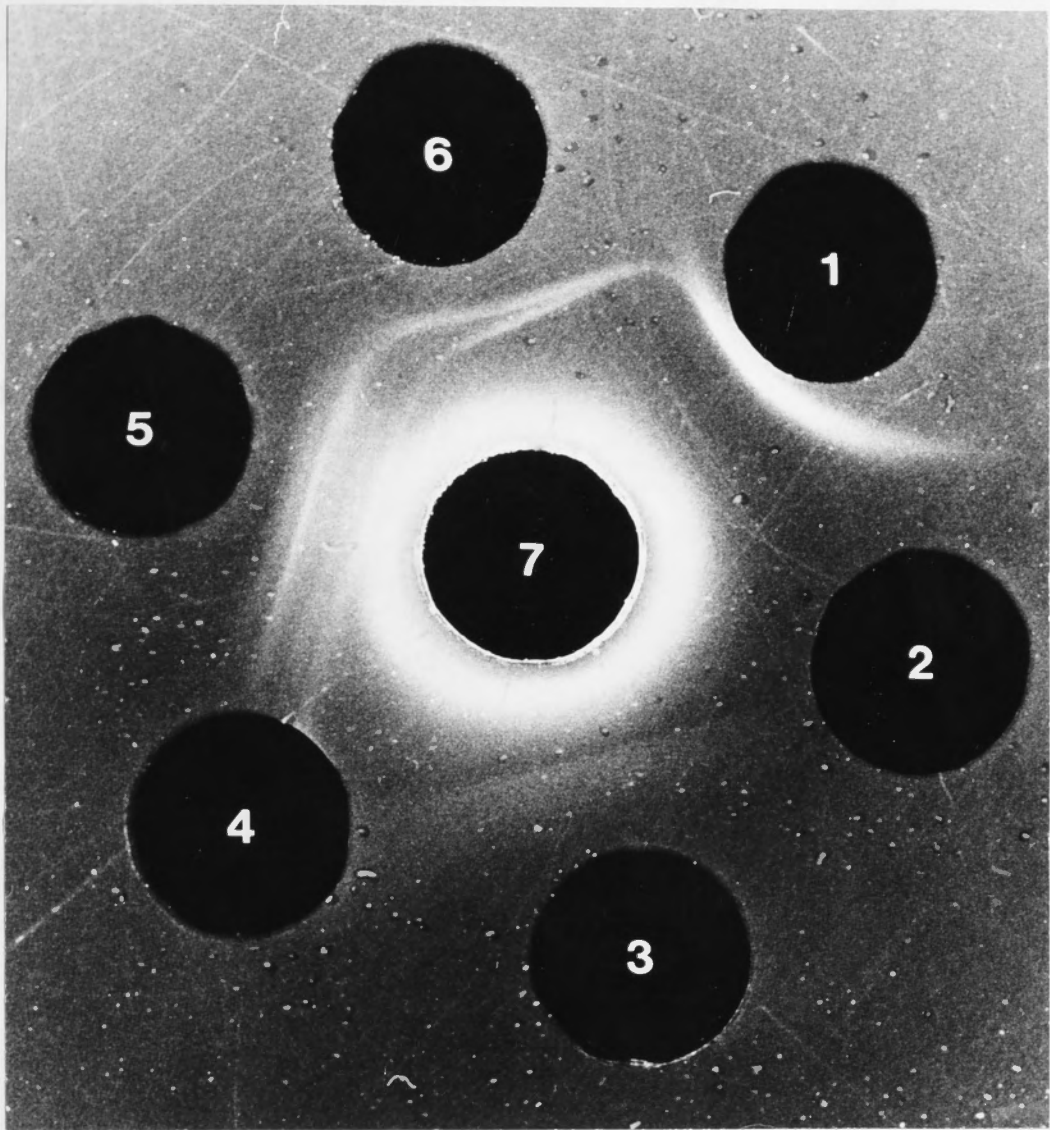
Confirmation of the double stranded nature of the material eluted from a cellulose column in the third peak was sought, by the specific property of dsRNA molecules to react to antiserum prepared specifically against dsRNA. This technique of investigation is particularly appropriate for small quantities of unlabelled dsRNA,

The procedure of immunodiffusion analysis used for testes dsRNA was identical to that previously described (section 3-2-6). Well 1 contained reovirus dsRNA; Wells 5 and 6, different preparations of poly(I).poly(C); Well 3, dsRNA isolated from rat testes, Well 4, heat denatured rat testes dsRNA. Well 2 was not used and Well 7 contains the antiserum specific for poly(I).poly(C). The result of the

FIGURE 4-10     Two dimensional immunodiffusion of antiserum to poly(I).poly(C) with various nucleic acid preparations

Antiserum was placed in the centre well. Outer wells contained 10  $\mu$ l of preparation in the amounts indicated; Well 1, Reovirus RNA (8.5  $\mu$ g); Well 3, dsRNA isolated from rat testes, (2  $\mu$ g); Well 4, the same preparation of rat testicular dsRNA, but heat denatured (2  $\mu$ g); Wells 5 and 6, different preparations of poly(I).poly(C) (1  $\mu$ g). Well 2, was not used. The 'halo' around the antiserum well was non-specific and is believed to be due to the diffusion of serum proteins.





immunodiffusion analysis is illustrated in Figure 4-10 and shows a conclusive band with the undenatured rat testes material, indicating reaction to a dsRNA molecule. This reaction disappears on heat denaturation; clear evidence for the presence of dsRNA in the rat testicular RNA.

#### 4-4 DISCUSSION

This chapter has dealt specifically with the origin of dsRNA. The two aspects dealt with involved, (1) cellular distribution, and (2) tissue distribution.

##### 4-4-1 *Cellular Distribution*

Having established that dsRNA existed within the rat liver cell, the next obvious question was the location. This question was investigated from several different approaches.

An obvious place for synthesis of dsRNA was the nucleus. This idea was first entertained as a result of the observation that labelled dsRNA appeared in nuclei as rapidly as nuclear RNA was labelled. The most rapidly labelled species of RNA are those which are being actively transcribed from the DNA within the nucleus. These include, in particular, the nuclear species such as nuclear precursors of tRNA, rRNA and mRNA. Although terminal addition of label to cytoplasmic tRNA was observed within a 20 min labelling period, very little, if any, of the labelled nuclear RNA species which do migrate, *viz* tRNA, rRNA and mRNA, will have migrated to the cytoplasm. Hence the discovery of an unreduced amount of labelled dsRNA species in a 20 min labelling experiment indicates a nuclear

origin for dsRNA. It does not exclude the possibility of a dsRNA species being synthesized at another site, e.g. in the mitochondria. There has been a recent suggestion that dsRNA is in part mitochondrial in origin (L. MONTAGNIER, personal communication).

Further evidence for a nuclear origin of dsRNA was presented. dsRNA was certainly discovered in the RNA prepared from isolated nuclei, but was consistently not detected in cytoplasmic RNA preparations free of nuclear RNA contamination. Following treatment of nuclear RNA with ribonucleases A and  $T_1$ , a distinct third peak fraction was obtained on cellulose chromatography. The double stranded nature of the third peak fraction has been categorically demonstrated. The general properties and detailed analysis of this fraction will be described in the following chapter.

It therefore appears that a nuclear origin has been firmly established for dsRNA, but an independent individual nuclear RNA species of dsRNA has not been demonstrated. Further work on this aspect will be considered in the next chapter. It seems likely that HnRNA or pure mRNA is a possible source of dsRNA. Experiments with low levels of actinomycin D clearly indicate that dsRNA is not derived from rRNA or ribosomal precursor RNA. Double stranded 'hairpin' regions of rRNA have been proposed as interspersed in the single stranded rRNA molecules (FRESCO *et al.* 1960; GOULD and SIMPKINS, 1969; COX, 1970). It appears unlikely, in view of the complete suppression of incorporation of



radioactive precursor into rRNA or pre-rRNA by actinomycin D, which does not affect the labelling of dsRNA, that these regions could contribute to the dsRNA isolated. Indeed the ribonuclease resistant fraction isolated from actinomycin D treated cellular RNA, has all the properties of dsRNA, and the percentage of the third peak material of the ribonuclease resistant RNA is unchanged.

Since tRNA or pre-tRNA contains only short segments of double stranded structure (6 or 7 nucleotide-pairs long, HOLLEY *et al.*, 1965) tRNA is not evidently a source of dsRNA in nuclei.

Overall, the results point to a nuclear origin for dsRNA. A nuclear origin has been recently suggested by other workers (KRONENBERG and HUMPHREYS, 1972); JELINEK and DARNELL, 1972; RYSKOV *et al.*, 1972; PATNAIK and TAYLOR, 1973). KRONENBERG and HUMPHREYS (1972), fractionated sea urchin embryos and discovered dsRNA in the nuclei, but did not achieve clean nuclei. There was as much as 10-20% cytoplasmic contamination. JELINEK and DARNELL (1972) used Hela cells and discovered that dsRNA could be isolated from high molecular weight RNA (> 45S) which suggested HnRNA as the origin for dsRNA. Their technique of identifying dsRNA did not include a heat-denaturation enzyme susceptibility profile, but was based solely on a caesium sulphate density estimation. RYSKOV *et al.*, 1972 also implicate "DNA-like RNA" (their expression for HnRNA) and pre-mRNA, as the source of a dsRNA which they isolate from Erhlich ascites tumour

cells by a hot-phenol, differential temperature extraction technique (GEORGIEV and MANTIEVA, 1962; GEORGIEV *et al.*, 1972). The 65° and 85°C fractions contain heavy (35S) intermediate (20-30S) and light (10-18S) pre-mRNAs (RYSKOV *et al.*, 1972). Two species of dsRNA were isolated, one from the heavy and one from the light, pre-mRNA regions. It is difficult to compare these two dsRNA species with the two rat liver dsRNA species, partly due to large differences in the technique of isolation. However, it does appear that a nuclear origin for at least one of these species could not be disputed. The work of PATNAIK and TAYLOR (1973), also reports the nucleus as the site of the ribonuclease resistant species, which they isolate. They describe also the isolation of two dsRNA species, one of which is a low molecular weight species similar to that described by JELINEK and DARNELL (1972), and which they conjecture may arise from HnRNA. The other species is, because of its sensitivity to actinomycin D, likely to be of viral origin. The purification of the dsRNA was not extensive, and the properties of the low molecular weight ribonuclease resistant species not extensively investigated.

Alternatively, preparations of cytoplasm free of nuclei were examined for dsRNA. The technique of preparation of the nuclei-free cytoplasm has been investigated and reported (NAORA and NAORA, 1967).

Whole cytoplasmic RNA was extracted and having treated the ribonuclease resistant RNA to analysis, a small percentage (0.3%) of the ribonuclease resistant material, was dis-

covered in the third peak on cellulose chromatography. This material did not show a typical dsRNA value on heat denaturation. More recently, in the light of experiments described in Chapter VII, and a mitochondrial origin, there may be a small part of this 0.3% which may represent a dsRNA of some significance and the lack of larger amounts of dsRNA should not be regarded as absolute evidence for the lack of a cytoplasmic species. Closer examination of cytoplasmic structures is warranted.

Collectively the evidence of this chapter has pointed to the nucleus as the site of biosynthesis of dsRNA. It appears to have a rapid labelling pattern similar to HnRNA. The actinomycin D resistant synthesis of dsRNA indicates the HnRNA or pre-mRNA regions as a likely origin for dsRNA. It does not mean necessarily that all dsRNA segments isolated are derived from part of the long HnRNA molecules. In fact, a particular species of dsRNA, other than part of the long HnRNA molecules, may exist in the nuclei. Recent experiments employing a "snap back" technique with HnRNA, showed approximately 70-90% recovery of a dsRNA fraction (RYSKOV *et al.* 1972; JELINEK and DARNELL 1972), and although technical difficulties may have been the reason for incomplete recovery, the above possibility could not be eliminated. This particular point will be elaborated in the following chapter.



#### 4-4-2 *Tissue Distribution*

The experimental evidence of this chapter describes the presence of dsRNA in other rat tissues. Since the metabolic patterns of RNA synthesis and the cellular functions of testes cells are different from liver cells, the rat testes was chosen as an alternative for analysis. Other tissues could be investigated, e.g. spleen or kidney. It appears however, that degradative enzymes released from the lysosomes, and in particular ribonucleases, are responsible for rapid breakdown of the RNA during homogenization before phenol extraction in both the spleen and kidney tissue. This difficulty is not apparent with rat testes, but two difficulties were noted with this tissue. One was the inability to label the cellular RNA sufficiently, and hence the dsRNA, which is such a small proportion of RNA, is insufficiently labelled to allow further analysis and detection with cellulose chromatography. The second was the presence of large amounts of DNA relative to the RNA content. The DNA constitutes a large proportion of the genetic material in testicular cells. Contamination with DNA in testicular RNA preparations was avoided by extensive treatment with deoxyribonuclease, followed by sucrose density gradient centrifugation. The cellular RNA, when treated with ribonucleases and chromatographed on cellulose, gives definite indications of containing dsRNA. Its presence was clearly confirmed by immunodiffusion analysis. dsRNA is therefore not singularly confined to the liver cell and is quite likely

CHAPTER V - PROPERTIES OF dsRNA ISOLATED FROM NUCLEI:  
MOLECULAR ORIGIN  
5-1 INTRODUCTION  
to be present in other rat tissues. Indeed dsRNA was found in a variety of uninfected cells (details and references are described in Chapter I). The presence of dsRNA therefore appears to be universal. It can be speculated that dsRNA(s) is involved in a mechanism of basic nuclear function. Its function, if it is universal, remains to be discovered.

~~The other point to notice is that the finding of dsRNA in other types of tissue renders even more unlikely a viral origin.~~

Identification of dsRNA is established by the observation of a sharp melting temperature at which there is a transition from ribonuclease resistance to ribonuclease sensitivity. Detailed gel electrophoresis analysis was used to determine the size, and to allow comparison with the dsRNA isolated from whole cells.

The second aim of the chapter is to provide detailed analysis as to which nuclear RNA contains the double stranded structure within the molecule, or whether it exists as an independent molecule, or both. This was achieved by fractionating the nuclear RNA using a sucrose gradient, and pooling individual fraction(s) according to the peaks representing the different molecular weight species. These fractions were then individually analyzed for dsRNA by testing the ribonuclease resistant RNA prepared from each fraction and purified by cellulose chromatography. The pooled third peak fractions from the chromatograms were then tested for loss of ribonuclease resistance on heat denaturation. A detailed analysis of the different

CHAPTER V - PROPERTIES OF dsRNA ISOLATED FROM NUCLEI:MOLECULAR ORIGIN5-1 INTRODUCTION

The purpose of this chapter is to provide details of the analysis of the particular dsRNA fraction isolated from nuclei. The first aim of the experiments was to confirm the double stranded nature of the material eluted from the third peak of cellulose column chromatography. Positive identification of dsRNA is established by the observation of a sharp melting temperature at which there is a transition from ribonuclease resistance to ribonuclease sensitivity. Detailed gel electrophoresis analysis was used to determine the size, and to allow comparison with the dsRNA isolated from whole cells.

The second aim of the chapter is to provide detailed analysis as to which nuclear RNA contains the double stranded structure within the molecule, or whether it exists as an independent molecule, or both. This was achieved by fractionating the nuclear RNA using a sucrose gradient, and pooling individual fraction(s) according to the peaks representing the different molecular weight species. These fractions were then individually analyzed for dsRNA by testing the ribonuclease resistant RNA prepared from each fraction and purified by cellulose chromatography. The pooled third peak fractions from the chromatograms were then tested for loss of ribonuclease resistance on heat denaturation. A detailed analysis of the different



molecular fractions of nuclear RNA can provide evidence as to whether dsRNA is part of a longer molecule on an independent species or both. Information like this may provide clues to the true biological significance of dsRNA itself.

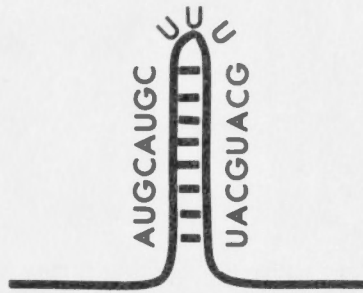
The use of a technique known as the "snap-back" technique, is illustrated in this chapter and schematically in Figure 5-1-a and b. The purpose of this method is to discover whether dsRNA species, which may exist as part of a longer single stranded RNA molecule, exist in an inter-molecular or intra-molecular form. If two different polynucleotide chains are linked by hydrogen bonds, this constitutes inter-molecular pairing, if different regions of the same polynucleotide chains are linked, this is intra-molecular. In most cases the intra-molecular form can be thought of as a "hairpin" loop in a single stranded RNA, where two linear adjacent base sequences are so closely matched that they form a hydrogen bonded structure to the extent of the complementary bases. This effectively has the appearance of a "hairpin" with a larger or smaller loop connecting the two sequences. To detect which type of bonding is present, the intact nuclear RNA, or nuclear RNA fraction, is heated at the denaturation temperature at which the two strands will separate; if a "hairpin" structure exists, the two matching sequences will be held together by the interconnecting loop. If it is an intra-molecular structure, the two will become completely separated. If the denatured preparation is rapidly cooled, the intra-molecular loop structure can rapidly reform ("snap-back"), because it

FIGURE 5-1-a    Schematic comparison between heat denatur-  
ation and subsequent ribonuclease treatment  
of inter- and intra-molecular bonded  
structures in nRNA molecular weight sub-  
classes

For each nuclear RNA subclass I → V

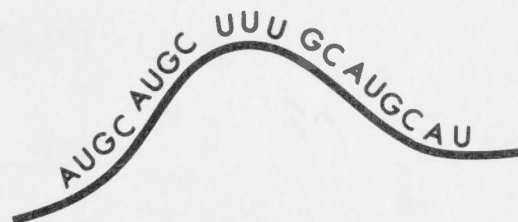
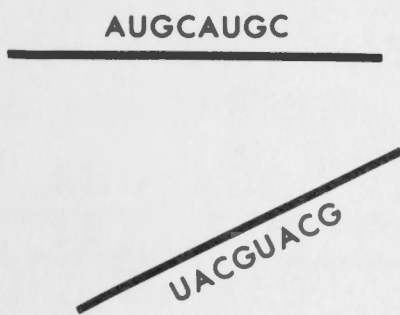


inter molecular hydrogen bonding

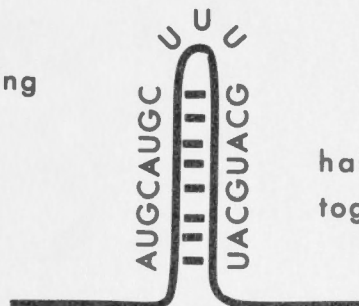
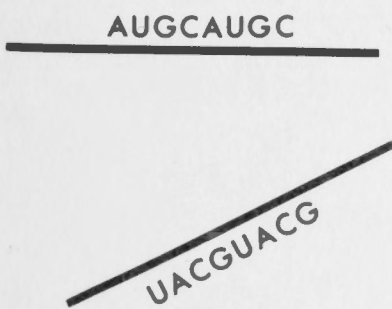


intra molecular hydrogen bonding  
'hairpin' structure

heat denaturation

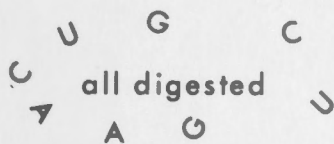


rapid cooling



hairpins reform held  
together by uuu loop

ribonuclease treatment

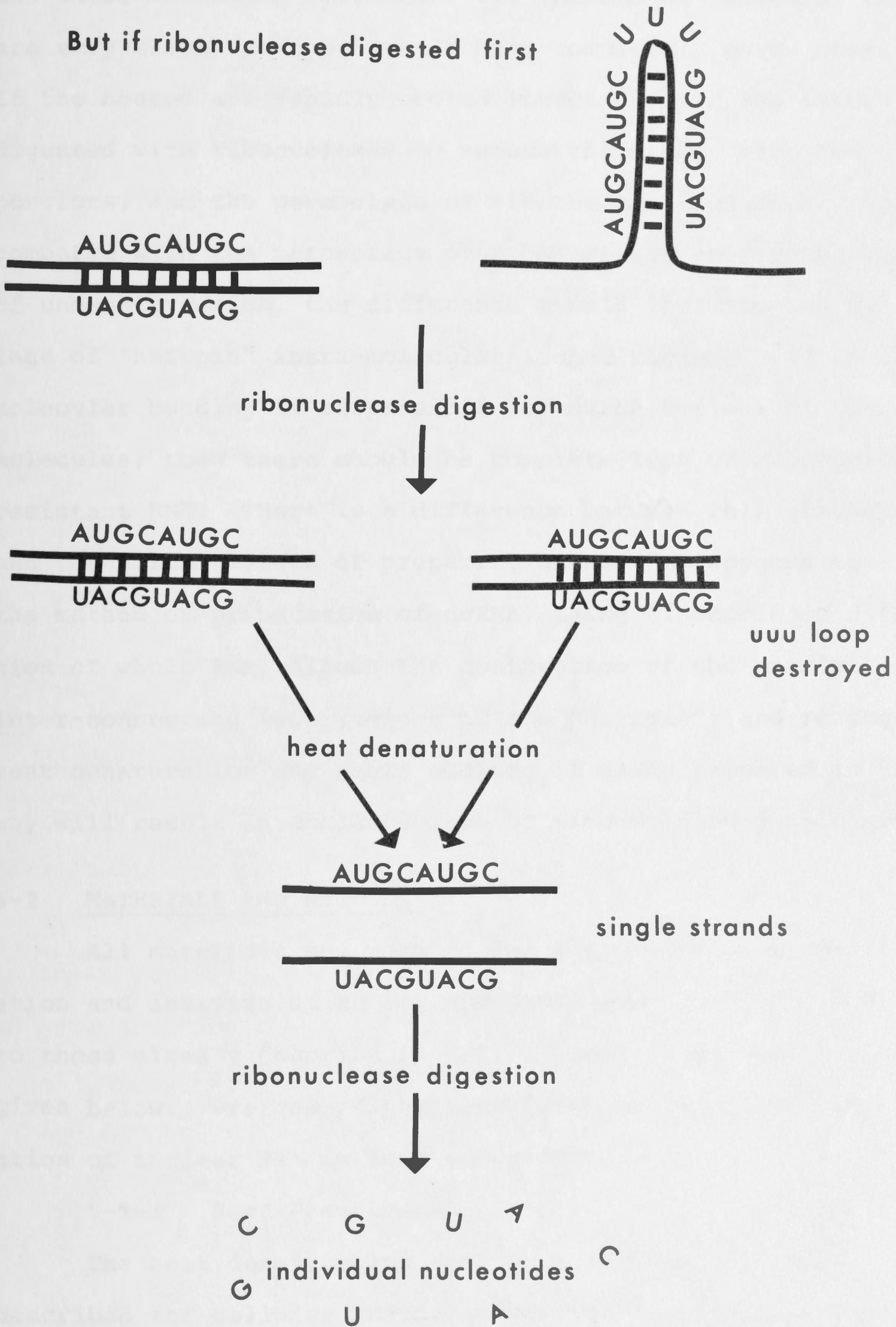


dsRNA hairpins intact



FIGURE 5-1-b    The same schematic comparison, but where  
the nRNA material has been ribonuclease  
digested before heat denaturation

But if ribonuclease digested first



is held in adjacent relative positions by the loop. In the inter-molecular situation, the chances of reforming rapidly are very slight as the strands have completely moved apart. If the heated and rapidly cooled intact nuclear RNA is now digested with ribonuclease to remove the single stranded portions, and the percentage of ribonuclease resistant regions compared with the percentage of ribonuclease resistant regions of undenatured RNA, the difference should indicate the percentage of "hairpin" intra-molecular looped regions. If inter-molecular bonding is responsible for dsRNA regions of longer molecules, then there should be complete loss of ribonuclease resistant RNA. There is a difference between this situation and the normal method of preparing dsRNA. It appears that the method of preparation of dsRNA, using ribonuclease digestion of whole RNA, allows the destruction of the possible inter-connecting loop regions of the "hairpin", and consequently heat denaturation and rapid cooling of dsRNA prepared in this way will result in complete loss of ribonuclease sensitivity.

## 5-2 MATERIALS AND METHODS

All materials and methods for the preparation, purification and analysis of nuclei and dsRNA fractions are identical to those already described. Only additional methods will be given below. Protease digestions were omitted in the purification of nuclear RNA in some experiments.

### 5-2-1 *Heat Denaturation*

The heat denaturation method is identical to that described for cellular RNA under section 3-2-3. Heat denaturation was conducted in low salt solution only, (0.1 x SSC).



Except where indicated, dsRNA was prepared from nuclear RNA which has not been purified using a protease digestion step.

#### 5-2-2 *Gel Electrophoresis*

Gel electrophoresis was carried out using 10% polyacrylamide gels in an identical manner to that described under section 3-2-7.

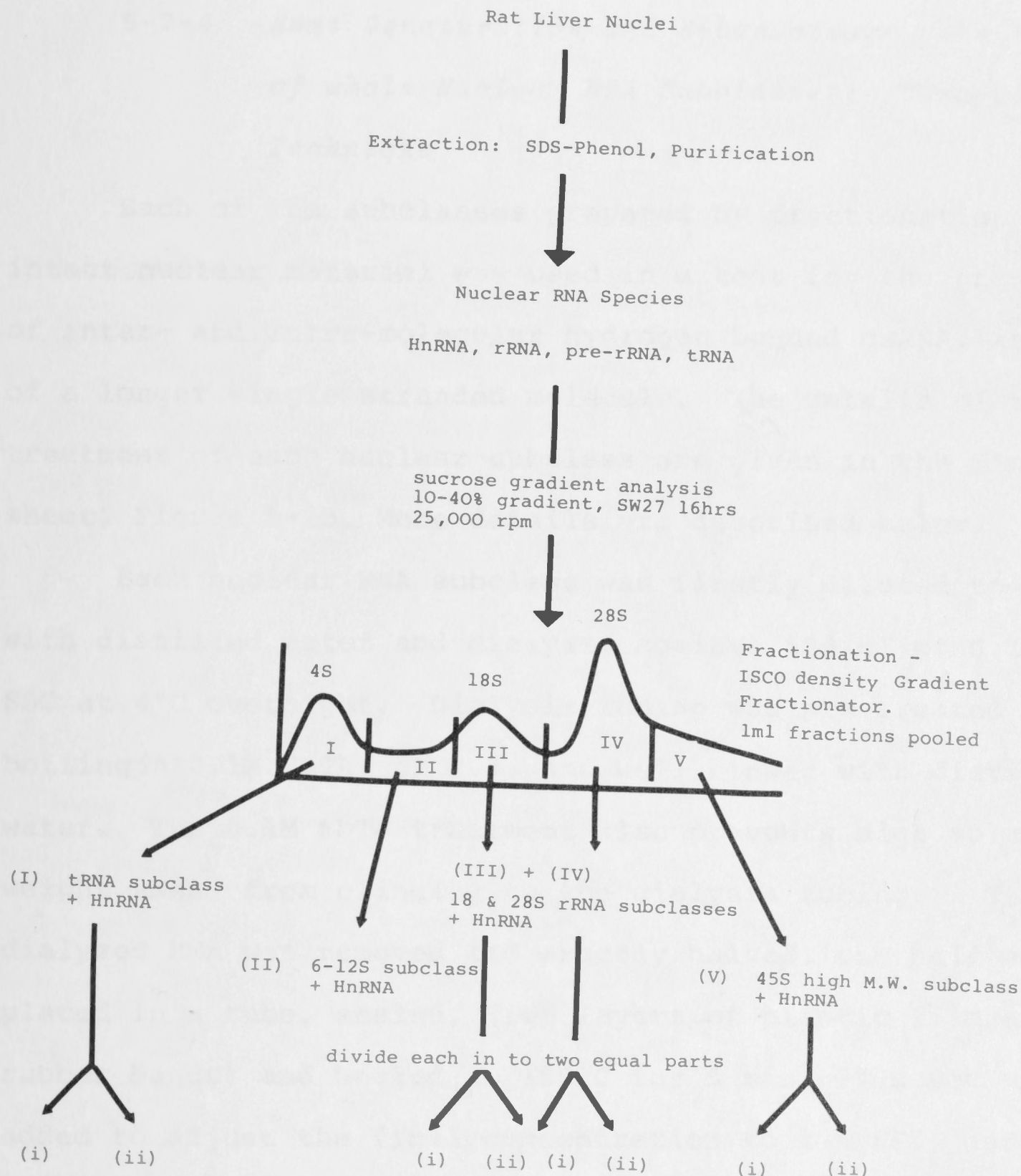
#### 5-2-3 *Sucrose Gradient Fractionation of Nuclear RNA*

The procedure for fractionation is illustrated diagrammatically in a flow sheet, Figure 5-2a. The details are described in the text below.

Nuclear RNA was prepared from isolated nuclei as described in the previous chapter. The undegraded  $^3\text{H}$ -labelled purified nuclear RNA preparation (2.6 mg of RNA dissolved in 1 ml of 10 mM Tris-HCl 0.14, NaCl buffer pH 7.4) was layered onto a 10-40% sucrose density gradient made in the buffer described above, in which the RNA was dissolved. Gradients were centrifuged in a Beckman SW27 swinging bucket rotor at 25,000 rpm for 16 h at 4°C. The gradients were then fractionated on an ISCO density gradient fractionator and 1 ml fractions collected. Fractions were pooled into five subclasses as indicated in section 5-3-3, each representing a region of nuclear species of different molecular weight. To these collective fractions two volumes of ethanol was added and each kept overnight at -20°C. These fractions were then centrifuged at 400 rpm in the SS-34 rotor of the RC2B Sorvall centrifuge (1940 x g). The precipitated RNA was

FIGURE 5-2-a    Schematic representation of the fraction-  
ation of nuclear RNA into discrete  
molecular weight subclasses

# FRACTIONATION





then washed once with ice-cold 80% ethanol and recentrifuged. Each final RNA precipitate (5) was dissolved in 0.5 ml of 10 mM Tris-HCl, 1 mM  $MgCl_2$ , 0.3M NaCl, pH 7.4, and stored at  $-20^{\circ}C$ .

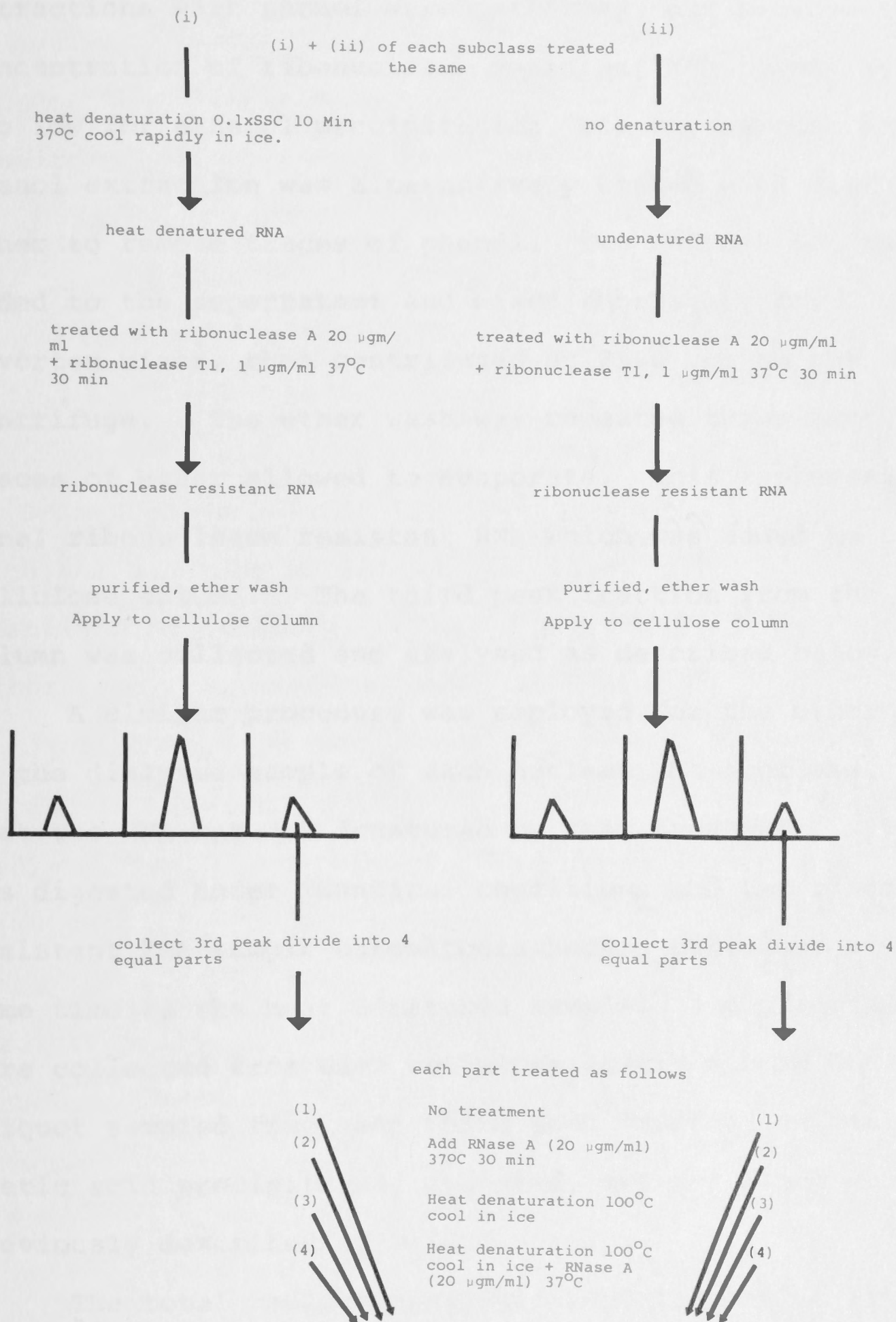
5-2-4     *Heat Denaturation and Ribonuclease Sensitivity  
of whole Nuclear RNA Subclasses: "Snapback"  
Technique*

Each of the subclasses prepared by fractionation of intact nuclear material was used in a test for the presence of inter- and intra-molecular hydrogen bonded dsRNA, as part of a longer single stranded molecule. The details of the treatment of each nuclear subclass are given in the flow sheet, Figure 5-2b. More details are described below.

Each nuclear RNA subclass was firstly diluted to 1 ml with distilled water and dialyzed against 500 ml of 0.1 x SSC at  $4^{\circ}C$  overnight. Dialysis tubing was pre-treated by boiling in 0.1M EDTA, pH 7.4, and well rinsed with distilled water. The 0.1M EDTA treatment also prevents high molecular weight RNAs from clinging to the dialysis tubing. The dialyzed RNA was removed and exactly halved, one half was placed in a tube, sealed, (two layers of plastic film and rubber bands) and heated to  $100^{\circ}C$  for 5 min, 20 x SSC was added to adjust the final concentration to 2 x SSC, and rapidly plunged into ice. Ribonuclease A (20  $\mu g/ml$ ) and ribonuclease  $T_1$  (1  $\mu g/ml$ ) were added and the mixture incubated at  $37^{\circ}C$  for 30 min. The mixture was then extracted with sodium dodecyl sulphate and phenol, using identical

FIGURE 5-2-b    Schematic representation of the treatment  
of each molecular weight subclass divided  
in two parts according to the fraction-  
ation procedure in Figure 5-2-a

# TREATMENT



Each precipitated Trichloroacetic acid dried and counted

Results given table 5-1



concentrations and conditions described for ribonuclease resistant RNA extractions (section 2-2-8). Two 10 min extractions with phenol were performed, but because the concentration of ribonuclease resistant RNA remaining was too low for ethanol precipitation, the supernatant after phenol extraction was alternatively washed with diethyl ether to remove traces of phenol. Two volumes of ether were added to the supernatant and mixed thoroughly for 2 min using a vortex mixer, then centrifuged at 2500 rpm in the bench centrifuge. The ether wash was repeated three times, and traces of ether allowed to evaporate. This represented the final ribonuclease resistant RNA which was added to a small cellulose column. The third peak fraction from the cellulose column was collected and analysed as described below.

A similar procedure was employed for the other half of the dialysed sample of each nuclear RNA subclass, except that the RNA was not denatured by heat treatment. The RNA was digested under identical conditions and the ribonuclease resistant RNA sample chromatographed on cellulose at the same time as the heat denatured sample. 3 ml fractions were collected from each cellulose column and an 0.5 ml aliquot sampled from each third peak fraction and trichloroacetic acid precipitated, filtered, and dried and counted as previously described (2-2-10).

The total radioactivity of the third peak fraction eluted from cellulose chromatography was obtained and

expressed as a percentage of the total radioactivity of the nuclear RNA subclass. These percentages, i.e. the percentage of dsRNA obtained from each, either heat denatured or undenatured nuclear RNA subclass, were compared to determine the effect of heat denaturation and consequently the percentage of "hairpin" structures in each nuclear RNA subclass.

5-2-5 *Confirmation of the Presence of a Double Stranded Structure in the Third Peak Fractions of Cellulose Chromatography of Digested Nuclear-RNA subclasses*

The third peak fractions of each cellulose column of heat denatured or undenatured nuclear RNA subclasses were tested for dsRNA by analyzing each third peak fraction for susceptibility to ribonuclease before and after further heat denaturation. An aliquot of the pooled third peak fraction was adjusted to 0.3M NaCl and divided into four equal parts. Two of these were heat denatured (100°C, 10 min - cooled rapidly in ice). One of the heat denatured and one of the undenatured samples were each treated with ribonuclease A (20 µg/ml) at 37°C, 30 min. All four samples were then trichloro-acetic acid precipitated, filtered, dried and counted as described under section 2-2-10.

5-3 RESULTS

5-3-1 *Melting Profile of Nuclear Derived dsRNA*

The material eluted in the third peak of cellulose chromatography of ribonuclease treated nuclear RNA was subjected to heat denaturation treatment. The main argument,

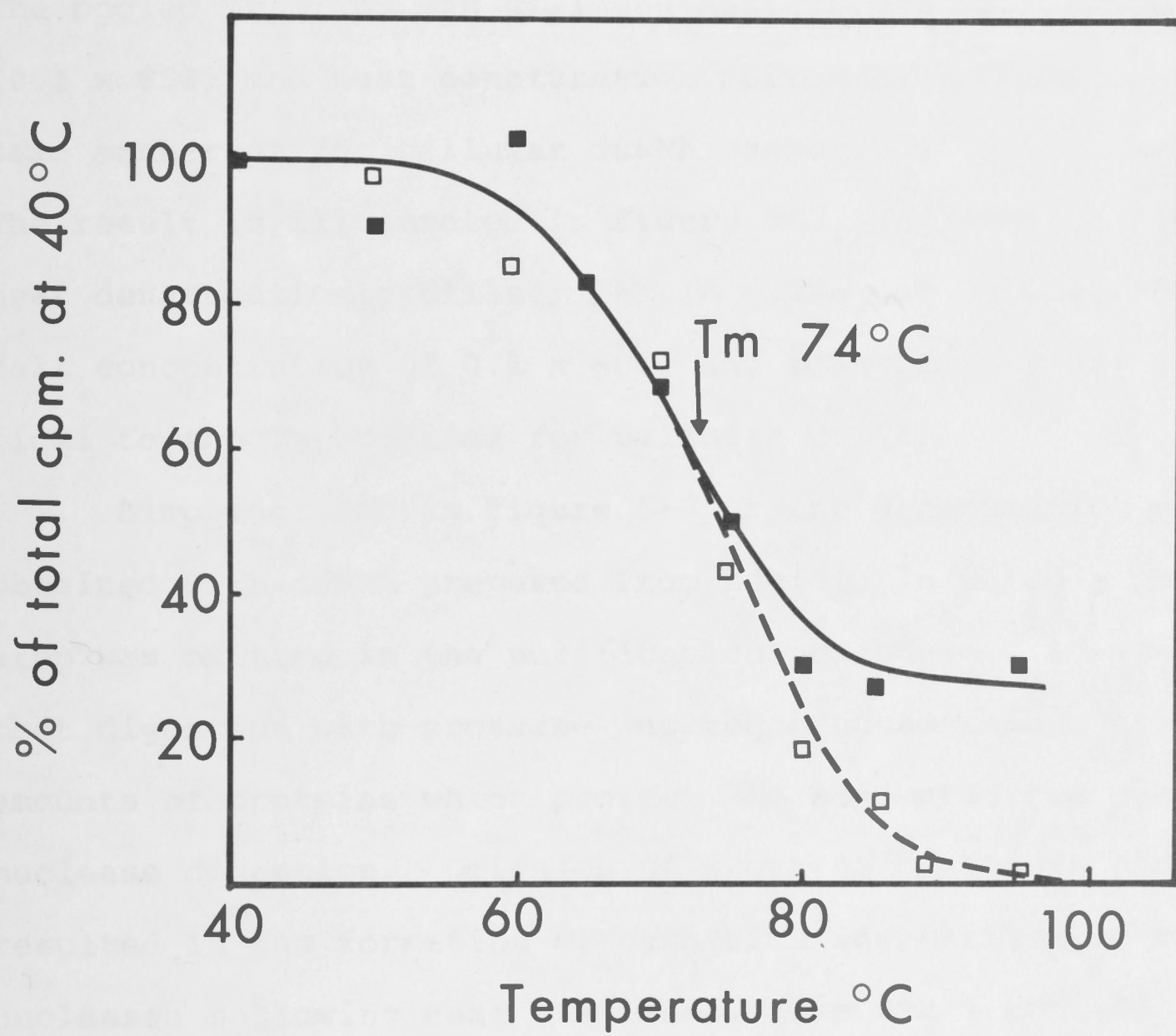
FIGURE 5-3     Heat denaturation profile of  $^3\text{H}$ -labelled  
dsRNA isolated from rat liver nuclei

dsRNA isolated from the third peak of cellulose chromatography of ribonuclease resistant RNA derived from  $^3\text{H}$ -labelled nuclear RNA, was heat denatured in 0.1 x SSC. The ribonuclease sensitivity at various temperatures was measured by ribonuclease A digestion in 2 x SSC (20  $\mu\text{g/ml}$ , 37°C, 30 min) and the acid-precipitable radioactivity remaining expressed as a percentage of the radioactivity at 40°C.

■————■ , Heat denaturation profile of a preparation of nuclear dsRNA in which protease had been omitted in the purification procedure.

□.....□ , The profile of similar dsRNA but in which protease had been employed in the purification procedure.





as with cellular dsRNA, that this fraction behaves as dsRNA, is its characteristic thermal denaturation profile.

Nuclear RNA was prepared from isolated whole nuclei and digested with both ribonucleases in high salt solution to remove single stranded RNA. The ribonuclease resistant RNA was chromatographed on cellulose and the third peak fraction pooled and concentrated, as previously described. The pooled fraction was dialyzed against low salt solution (0.1 x SSC) and heat denaturation performed in exactly the same manner as for cellular dsRNA, described in section 3-2-3. The result is illustrated in Figure 5-3, and shows a biphasic heat denaturation profile. The  $T_m$  value was 74°C at this low salt concentration of 0.1 x SSC, and consequently was identical to the  $T_m$  obtained for cellular dsRNA.

Also included in Figure 5-3 is the denaturation profile obtained with dsRNA prepared from nuclei, in which a protease step was omitted in the purification procedure. It was found that digestion with protease was required to remove trace amounts of proteins which protect RNA segments from ribonuclease digestion. Omission of protease treatment apparently resulted in the formation of complexes insensitive to ribonucleases following heat treatment. The trace proteins do not, however, alter the  $T_m$  value of the dsRNA, but there is a small percentage of radioactivity remaining after heat denaturation treatment if protease is not employed.

#### 5-3-2 *Gel Electrophoretic Analysis*

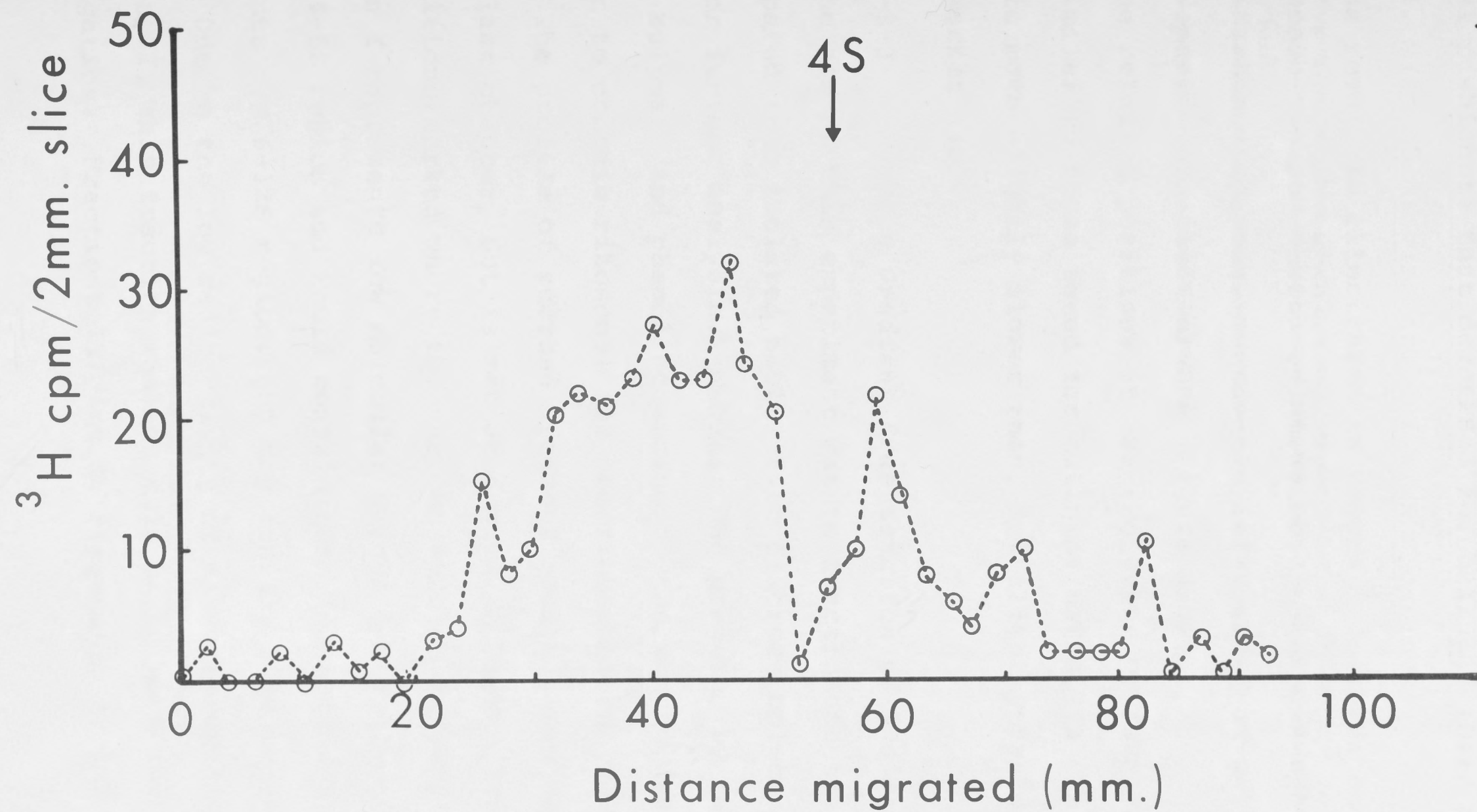
Gel electrophoretic analysis was carried out on a sample of third peak material chromatographed on cellulose.

FIGURE 5-4    Migration of dsRNA isolated from  $^3\text{H}$ -labelled  
nuclear RNA in 10% polyacrylamide gels

dsRNA was prepared from the third peak cellulose chromatographed material of ribonuclease resistant RNA from  $^3\text{H}$ -labelled rat livers. Gels were sliced into 2 mm slices and assayed for radioactivity. The position of rat liver tRNA applied as a marker (4S) is indicated.

○.....○ , Radioactivity/2 mm slice.





Samples were prepared for analysis and this was done in an identical fashion to that described for cellular third peak material.

The result ~~is~~ illustrated in Figure 5-4, ~~which~~ showed a heterogeneous distribution which is similar to that observed with dsRNA prepared ~~the presence of both a heterogeneous broad peak and a homo-~~  
~~geneous fast moving peak, as was similarly observed with~~  
~~dsRNA prepared from cellular RNA~~ (Figure 3-5).

The relative positions of the two types of dsRNA appear to be similar to those found for cellular dsRNA, in that they appear to move slightly slower than, and slightly faster than, a tRNA marker (4S).

#### 5-3-3 *Sucrose Gradient Fractionation of Nuclear RNA*

The aim of this experiment was to fractionate the nuclear RNA prepared from isolated nuclei into discrete molecular sizes for further analysis. Nuclear RNA prepared by sodium dodecyl sulphate and phenol according to the method described, is known to contain ribosomal and pre-ribosomal RNA, tRNA and HnRNA. The profile of sucrose gradient analysis was presented in the last chapter, but is repeated here in Figure 5-5, with the positions marked where the various subclasses were taken. Fraction I represents low molecular weight RNA sedimenting in the 4-5S region and would contain tRNA. Fraction II represents the 6-12S region, and may contain some pre-mRNA or mRNA. Due to the low radioactivity of material obtained in Fraction II, this fraction was not able to be used for further investigation. Fractions III and IV represent, in the majority,

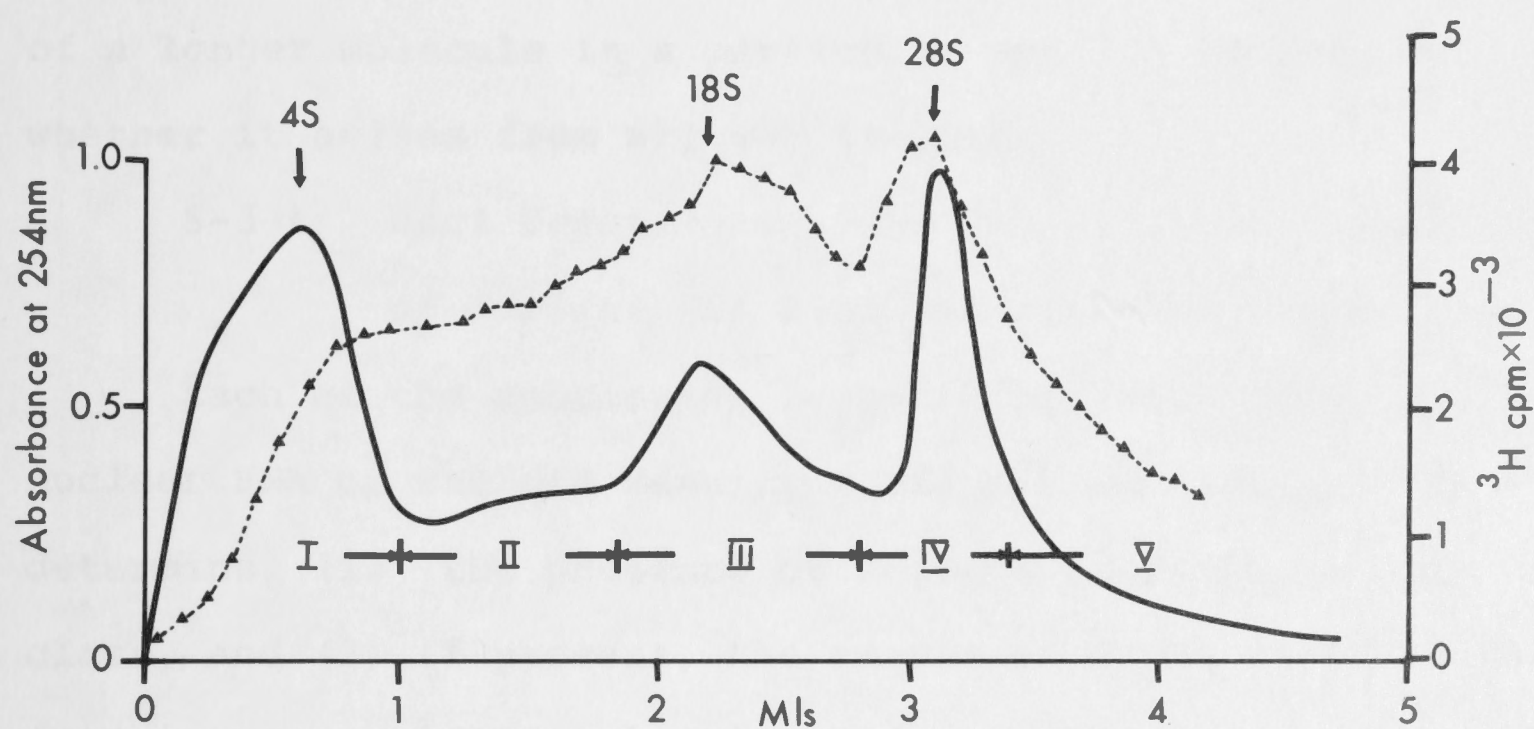
FIGURE 5-5    Sucrose gradient profile of  $^3\text{H}$ -labelled rat  
liver nuclear RNA

The profile indicated is identical to that described in Figure 4-2, however the position of the fractionation in molecule weight subclasses is indicated by I, II etc.

\_\_\_\_\_ , Absorbance at 254 nm

▲.....▲ , Radioactivity





the 18 and 28S peaks of ribosomal RNA, fraction V represents material larger in size than 45S, such as long HnRNA and pre-rRNA regions. As shown in Figure 4-6 in the previous chapter, HnRNA, which is insensitive to low doses of actinomycin D, is present over the whole range of molecular sizes, and indeed is the reason for the name, 'heterogeneous nuclear RNA'.

This fractionation of nuclear RNA into subclasses then allows analysis to determine whether dsRNA arises from part of a longer molecule in a particular species of RNA, or whether it arises from all sub fractions.

#### 5-3-4 *Heat Denaturation and Ribonuclease Sensitivity of Nuclear RNA Subclasses; "Snap-back" Analysis*

Each of the subclasses prepared by fractionation of nuclear RNA on sucrose density gradients was analyzed to determine, (1) the presence or absence of dsRNA in each subclass, and (2) if present, the amount of dsRNA which arises from inter- or intra-molecular hydrogen bonding. These two aims were achieved by the experimental procedures described above (5-2-4 and 5, and illustrated diagrammatically in Figures 5-1 and 5-2). The two aims were combined in practice and the result of an extensive series of experiments are presented in Table 5-1 and 5-2. Table 5-1 shows the amount of dsRNA in nuclear subclasses observed before and after heat denaturation of the original material. The profile of cellulose chromatography for each subclass of nuclear RNA after ribonuclease digestion was almost identical, and also the same as the general profile for unfractionated nuclear RNA

TABLE 5-1

Heat-treatment of nRNA subclasses prior to  
isolation of dsRNA

Sub- class of nRNA	Heat treated (100°C, 0.1 x SSC, 10 min, adjusted 2 x SSC) and cooled to 0°C			Untreated		
	Total cpm	cpm of third peak cellu- lose fraction	% third peak fraction of total cpm	Total cpm	cpm of third peak cellu- lose fraction	% third peak fraction of total cpm
I	197,750	81	0.04%	197,750	522	0.2%
II		Not analyzed				
III	500,000	796	0.16%	500,000	1,548	0.31%
IV	242,500	249	0.1%	242,500	621	0.25%
V	413,370	1896	0.45%	373,370	1,596	0.45%



TABLE 5-2

Rinonuclease sensitivity of third peak material obtained from heat treated and untreated subclasses of nRNA.

Subclass of nRNA	Heat Treatment of nRNA before isolation of dsRNA	Ribonuclease sensitivity of third peak			
		No RNase treatment cpm	No RNase but heat-denatured cpm	RNase treatment cpm	Heat-denaturation followed by RNase treatment cpm
I	Heat denatured	-	-	-	-
	Undenatured	78	98	30	15
II	Not analysed				
III	Heat denatured	116	130	36	26
	Undenatured	323	238	62	7
IV	Heat denatured	65	65	25	2
	Undenatured	125	79	52	12
V	Heat denatured	209	220	175	34
	Undenatured	222	218	167	56

#### 5-4 DISCUSSION

The existence of dsRNA in the RNA isolated from whole nuclei, has been substantially confirmed by the presence of a heat denaturation profile, showing the characteristic

given in the previous chapter (Figure 4-3). Table 5-2 shows the heat denaturation enzyme susceptibility analysis, indicating characteristic dsRNA behaviour.

It is apparent that each subclass of nuclear RNA does contain dsRNA. dsRNA can be isolated from all of the nuclear RNA subclasses, whether or not they are pretreated by heating and rapid cooling. This would argue that all nuclear RNA subclasses contain intra-molecular hydrogen bonded regions ("hairpins"). In the undenatured material, the content of dsRNA ranges from 0.25% to 0.45%, depending upon the subclass. The range in heat denatured material is from 0.04% to 0.45%. The result indicates that all of the subclasses of HnRNA, both pre-heat treated and untreated, before preparation of dsRNA, contain dsRNA. It should be mentioned that no difference in dsRNA content was observed between heat treated and untreated nRNA of subfraction V, indicating that in high molecular weight nRNA, all of the dsRNA appears to be derived from intra-molecular "hairpin" regions. In subclass I, (the low molecular weight nRNA) however, very little RNA appears to be derived from "hairpin" regions (0.04% compared with 0.26%). In subclasses III and IV, the percentages of "hairpins" contributing to dsRNA appears to be about 50%.

#### 5-4 DISCUSSION

The existence of dsRNA in the RNA isolated from whole nuclei, has been substantially confirmed by the presence of a heat denaturation profile, showing the characteristic

biphasic curve of sensitivity to ribonuclease digestion. In addition, the  $T_m$  value of nuclear dsRNA was identical to that obtained for cellular dsRNA. The size and gel electrophoretic patterns of dsRNA derived from nuclear RNA was also almost exactly the same as the cellular dsRNA profile. These two findings seemingly indicate that the dsRNA of nuclear origin is the same as the cellular dsRNA, and adds further support to the argument that dsRNA is substantially derived from the nucleus of the cell. There is still a possibility, however, of a small undetected dsRNA region associated with a cytoplasmic organelle, as has already been mentioned.

If a nuclear origin for dsRNA is accepted, the question we then asked was, which type of nuclear RNA contains the dsRNA regions as part of a longer single-stranded molecule, or whether dsRNA exists as an independent nuclear species. The investigations detailed in the preceding chapter, have already excluded the possibility of ribosomal and pre-ribosomal origin of dsRNA. The finding that the size of dsRNA molecules of nuclear origin are identical to those of cellular origin, excludes tRNA as a source of dsRNA, since the double stranded regions found in tRNA molecules are at the most 6-7 nucleotide pairs long, (HOLLEY *et al.*, 1965). This leaves HnRNA as the source of dsRNA, unless the dsRNA exists as an independent molecule. In the experiments described, dsRNA was found to be present in all subclasses of nRNA, supporting the evidence that HnRNA is the source



of dsRNA isolated, in the knowledge that HnRNA sediments over the whole range on a sucrose density gradient.

The highest proportion of dsRNA isolated from the fractionated nuclear species was found in the high molecular weight fraction (0.45% in fraction V). "Snap-back" analysis reveals that all of the dsRNA present in the high molecular weight fraction would appear to arise from the "hairpin" structures. Apparently all "hairpin" regions in the high molecular weight RNA have a chance to rapidly reform after denaturation. A similar experiment was conducted recently by JELINEK and DARNELL (1972) using Hela cell nuclear RNA of high molecular weight. Their results reveal that dsRNA is contained in the "hairpin" structures but showed only 90% recovery after heat denaturation. Their conditions for ribonuclease treatment to detect dsRNA were very mild (2  $\mu$ g/ml of ribonuclease A for 1 min at 37°C). As described in Chapter II, such mild treatment is not sufficient to quantitate the amount of dsRNA. The value of recovery would therefore seem to be an over-estimate. In contrast, the ribonuclease conditions used in the experiments described in this thesis, were 20  $\mu$ g/ml ribonuclease A with 1  $\mu$ g/ml of ribonuclease T<sub>1</sub> at 37°C for 30 min. RYSKOV *et al.*, (197<sup>2</sup>~~1~~) have also analyzed their two dsRNA fractions which they claim arise from pre-mRNA regions of RNA, and also come from "hairpin" regions. They report that 70% of the longer dsRNA regions survive heat denaturation and hence arise from "hairpins" and 98% are "hairpins" of the shorter dsRNA type. In these "snap-back"

experiments, precise characterisation of dsRNA obtained has not been carried out. Consequently, their results were not conclusive. The experiment described in this chapter reveal, however, that the material observed in the present "snap-back" experiment is certainly dsRNA and that 100% of the dsRNA derives from "hairpin" regions of the high molecular weight ( $> 45S$ ) Hn RNA. In low molecular weight nRNA, the recovery is only 15-50%. Such a low recovery may result from the presence of nicked or degraded RNA in the subfractions and/or from the existence of inter-molecular hydrogen bonded dsRNA, which is not derived from HnRNA. If the latter is the case, then subfraction I would be the likely fraction to contain an independent molecule because gel electrophoretic analysis reveals the size of dsRNA to be in the lower molecular weight region (4S). That does not necessarily mean that the size of such an independent molecule corresponds exactly to the peaks illustrated in the gel electrophoretogram, since the majority of the dsRNA analysed on gels is likely to have originated from the higher molecular weight HnRNA "hairpin" regions. It is possible, that the smaller, more homogeneous peak, contains the independent dsRNA molecule.

From the percentage of dsRNA recovered in the high molecular weight HnRNA fraction, it is possible to estimate the number of dsRNA regions in each HnRNA molecule. The size of the HnRNA molecule is reported as being between 10,000 and 20,000 nucleotides in length in the high molecular

weight fraction of HnRNA sedimenting between 40 and 70S (JELINEK and DARNELL, 1972; MOLLOY *et al.*, 1974). If one accepts 0.45% as the percentage of dsRNA in the high molecular weight HnRNA fraction, then the size of dsRNA would lie between 45 and 90 nucleotides. From the size calculated from the behaviour of dsRNA on polyacrylamide gels, it appears that only 1 base paired loop would be possible per HnRNA length. 0.45% is considerably less than 3% predicted by JELINEK and DARNELL, (1972), however, RYSKOV *et al.*, (1973) report figures of between 0.8 and 1.2% for the percentage of dsRNA in high molecular weight HnRNA, which would suggest 1 or 2 dsRNA regions per HnRNA length. It is also extremely difficult to exclude the possibility that degradation may affect the percentage recovery of dsRNA in the lower molecular weight subclasses. However, it is unlikely that the recovery would be affected in the higher molecular weight species. It is likely that the higher 3% value observed by JELINEK and DARNELL (1973) may be due to the mild digestion conditions employed, and the lack of adequate purification or characterization of their dsRNA species. The importance of the finding of 1 dsRNA region per HnRNA molecule will be discussed in Chapter VII.



CHAPTER VI - COEXISTENCE OF POLY(A) SEGMENTS WITH THE dsRNAFRACTION6-1 INTRODUCTION

Recently the attention of a large number of workers has been focused on the existence and properties of a length of polynucleotide containing only adenylic acid residues, commonly called poly(A). It is not the intention of this thesis to consider poly(A) in detail, but a brief resumé will be given here of the important findings of the poly(A) story in an endeavour to highlight possibilities of a dsRNA-poly(A) involvement.

The interest in poly(A) has been enhanced by the discovery that poly(A) sequences are contained in both the HnRNA and mRNA molecules. It now appears that mRNA in mammalian cells is derived from post-transcriptional modifications of longer RNA precursor molecules (DARNELL, JELINEK and MOLLOY, 1973). This is substantiated by the findings that both HnRNA and mRNA contain a discrete poly(A) segment; and that when cells are briefly exposed to labelled adenosine, the labelled poly(A) obtained from HnRNA and mRNA are of identical size as determined by gel electrophoresis (SHIENESS and DARNELL, 1973). Further evidence has indicated the poly(A) segment of both mammalian mRNA and HnRNA exists at the 3'OH end of these molecules, and would appear to be approximately 200 nucleotides in length (MENDECKI, LEE and BRAWERMAN, 1972). Further investigations have revealed that the poly(A) is added post-transcriptionally (DARNELL *et al.*, 1971; PHILIPSON *et al.*, 1971; BIRNBOIM, 1973) and appears to be added stepwise to the HnRNA

sequences (JELINEK *et al.*, 1973). It is apparent from the work using the specific inhibitor, cordycepin, used to terminate RNA chains prematurely (PENMAN, ROSBACH, PENMAN, 1970), that not only is rRNA synthesis stopped in the nucleus, but also the addition of poly(A) segments is prevented, and mRNA then fails to appear in the cytoplasm (DARNELL *et al.*, 1971; JELINEK *et al.*, 1973; MENDECKI, LEE and BRAWERMAN 1972). While the precise role of poly(A) is still unknown, it appears increasingly possible that it may be closely involved in the regulation of gene expression in higher organisms, in particular in control at the post-transcriptional level. Possible models of regulation of gene expression are adequately described by DARNELL, JELINEK and MOLLOY (1973).

The first indication of the possibility of poly(A) segments contaminating dsRNA preparations, was a discovery that base analysis of the third peak fraction of cellulose chromatography of cellular ribonuclease resistant RNA, revealed a high value for A in the analysis, whereas for dsRNA equal values of A and U would be expected in a paired molecule (see Chapter III). Poly(A) has the property of being resistant to digestion with ribonucleases, and therefore for this reason also may be suspected as a likely contaminant of unpurified ribonuclease resistant material.

Poly(A) segments have another property, and that is a capacity to bind to unsubstituted cellulose. Poly(A) segments also bind to millipore filters under high salt conditions (LEE, MENDECKI and BRAWERMAN, 1971; MENDECKI, LEE and

BRAWERMAN, 1972). Binding of poly(A) segments to unsubstituted cellulose has been investigated by KITOS *et al.*, (1972); SCHUTZ *et al.*, (1972); DELARCO and GUROFF (1973) and SULLIVAN and ROBERTS (1973). Poly(A) will also bind well to oligo-(dT) cellulose, i.e. cellulose with 5'deoxythymidilic acid residues covalently bound to it (GILHAM, 1964; EDMONDS, 1971; FAUST *et al.*, 1973). Most recently the particular binding capacities of poly(A) segments and the ionic conditions and temperature factors of binding to unsubstituted and oligo-(dT) cellulose have been thoroughly investigated (MERCER, 1974).

6-2 The capacity to bind to unsubstituted cellulose has important implications in the technique used for dsRNA preparation, as cellulose chromatography constitutes the major purification procedure. The preferential binding of poly(A) segments to millipore filters (LEE, MENDECKI and BRAWERMAN, 1971; MENDECKI, LEE and BRAWERMAN, 1972), and to oligo-(dT) cellulose (GILHAM, 1964; EDMONDS, 1971; and FAUST *et al.*, 1973; MERCER, 1974) however, can be used to remove poly(A) segments from dsRNA preparations.

used The aims of the experiments in this chapter are to investigate to what extent poly(A) segments may be contaminating the preparation of dsRNA. This in turn involves some consideration of the behaviour of poly(A) under the conditions used for cellulose chromatography and preparation of dsRNA. The presence of poly(A) in the third peak of cellulose chromatography is investigated using base analysis, millipore binding, and oligo-(dT) cellulose.



In addition, because of the importance of poly(A) segments in the understanding of biogenesis of mRNA, and in the regulation of protein synthesis and the involvement of poly(A) in post-transcriptional events, a possible relationship between poly(A) segments and dsRNA is investigated, i.e. whether there is a covalent linkage existing between dsRNA and poly(A). Both the poly(A) binding to oligo-(dT) cellulose, and the material which passes through without binding to an oligo-(dT) cellulose column, is examined for dsRNA characteristics. Possible implications of such a union are considered in the Discussion.

## 6-2 MATERIALS AND METHODS

As previously stated, all materials and methods are identical to those used in previous chapters. Only those extra methods pertaining to this chapter are given below.

### 6-2-1 *Materials*

Synthetic poly adenylic acid was purchased from Miles Laboratory, Inc. Elkhart, Ind., 2'Deoxythymidine 5'-phosphate was purchased from Sigma Chemical Co. and unsubstituted cellulose was Whatman CF-11 cellulose. Millipore filters used were

## METHODS

### 6-2-2 *Oligo-(dT) Cellulose Column Chromatography*

Oligo-(dT) cellulose was synthesized by the method of GILHAM 1964, using Whatman CF-11 cellulose which had been washed according to the method of PERRIN (1966) and dried

*in vacuo*  $P_2O_5$  at 45°C. In addition to the washing procedures in the above reference (GILHAM 1964), the product was washed with 0.1M NaOH. The oligo-(dT) cellulose was kindly provided by Dr. J.F.B. Mercer in our laboratory. Small columns (1 cm x 4 cm) were prepared with this cellulose and equilibrated with 20 mM Tris-HCl, 1 mM  $MgCl_2$  and 0.5M NaCl buffer pH 7.4.  $^{32}P$ -labelled third peak material to be applied was dissolved in the same buffer. Fractions of 1 ml were collected, precipitated and counted, as described in 2-2-10. Retained material was removed by eluting with distilled water, and fractions were collected and treated similarly to assay radioactivity.

#### 6-2-3 Millipore Filter Method of Binding poly(A)

##### *Segments*

The millipore filter binding method used was as described by LEE, MENDECKI and BRAWERMAN, 1971 and MENDECKI, LEE and BRAWERMAN, 1972). Essentially the methods consist of dissolving the  $^{32}P$ -labelled RNA samples in 10 mM Tris-HCl, 1 mM  $MgCl_2$  and 0.5M KCl, pH 7.6., and applying the ice-cold mixture to millipore filters (0.45  $\mu$  pore size) soaked previously in the same ice-cold solution for 30 min and filtering slowly. For radioactivity assays the filters were heat dried and 10 ml of toluene scintillation fluid added to each and counted.

#### 6-2-4 Gel Electrophoresis

Gel electrophoresis was performed in an identical manner to that previously described, section (3-2-7).

### 6-3 RESULTS

#### 6-3-1 *Base Analysis: Presence of Poly(A)*

The base composition of  $^{32}\text{P}$ -labelled cellular double stranded fraction eluted in the third peak of cellulose column chromatography was presented in Table 3-1 and is given also in Table 6-1. The method of base analysis is described in detail in Chapter III. The material is characterized by a low G + C content, but surprisingly high adenylic acid content. The excess of adenylic acid residues, over what would be expected if A = U, is 12.5% as determined from the actual base analysis figures. There are two methods for determining the percentage of poly(A) segments contained in the material isolated in the third peak of cellulose chromatography. They are oligo-(dT) cellulose binding of poly(A), and millipore filter analysis. Both these methods indicate a value of 12% of poly(A) contamination. Consequently the base composition is corrected for poly(A) content by taking this 12% figure into account. Because the same figure was obtained by three different methods of analysis, it is reasonable to correct the actual base analysis figures by 12%, and these corrected values are indicated in Table 6-1.

Since long poly(A) sequences are present in mRNA and HnRNA molecules, there exist three possibilities for their location. Either the dsRNA contains a number of short poly(A) segments within its molecular structure, or long poly(A) is bound to the end of the dsRNA. Alternatively the



TABLE 6-1 [This table is a repeat of Table 3-i]

Base composition of <sup>32</sup>P-labelled ribonuclease resistant whole liver cellular RNA isolated from the third eluate of cellulose column chromatography. The values shown are the average of three determinations.

Base composition (moles percent)					G + C content (%)	G/C	A/U
	A	G	C	U			
Measured	37.5 ± 1.7	18.4 ± 1.2	18.9 ± 1.5	25.0 ± 1.8	37.3	0.97	1.50
Corrected	28.2	21.2	21.8	28.8	43.0	0.97	0.98

Purified <sup>32</sup>P-labelled ribonuclease resistant RNA was prepared in the normal way and chromatographed on the small cellulose column. A 2.5 ml aliquot of each 3 ml fraction eluted in the third peak was dialyzed against the buffer 10 mM Tris-HCl, 1 mM MgCl<sub>2</sub> and 0.5M KCl, pH 7.6, and treated according to the millipore filter analysis described above. These results are presented in Figure 6-1, and demonstrate that poly(A) was indeed present in the third peak. The total radioactivity of the material in each third peak fraction was determined after trichloroacetic acid precipitation of the remaining 0.5 ml (method described in section 2-2-10). The

poly(A) itself eluted coincidentally in the third peak on cellulose.

6-3-2 *Millipore Analysis of the third Peak Fraction of Cellulose Chromatography*

Material eluting in the third peak from cellulose columns has already been shown to be dsRNA. However, in view of the results obtained by base analysis, there appeared to be some contamination with adenylic acid. Consequently this material was examined for poly(A) content by the millipore technique (LEE, MENDECKI and BRAWERMAN, 1971; MENDECKI, LEE and BRAWERMAN, 1972). Any poly(A) segments contained in the third peak fraction bind preferentially to the millipore filters, where high KCl concentration is used. Poly(A) segments will remain on the filters, whereas the dsRNA would pass through the filters.

Purified  $^{32}\text{P}$ -labelled ribonuclease resistant RNA was prepared in the normal way and chromatographed on the small cellulose column. A 2.5 ml aliquot of each 3 ml fraction eluted in the third peak was dialyzed against the buffer 10 mM Tris-HCl, 1 mM  $\text{MgCl}_2$  and 0.5M KCl, pH 7.6, and treated according to the millipore filter analysis described above. These results are presented in Figure 6-1, and demonstrate that poly(A) was indeed present in the third peak. The total radioactivity of the material in each third peak fraction was determined after trichloroacetic acid precipitation of the remaining 0.5 ml (method described in section 2-2-10). The

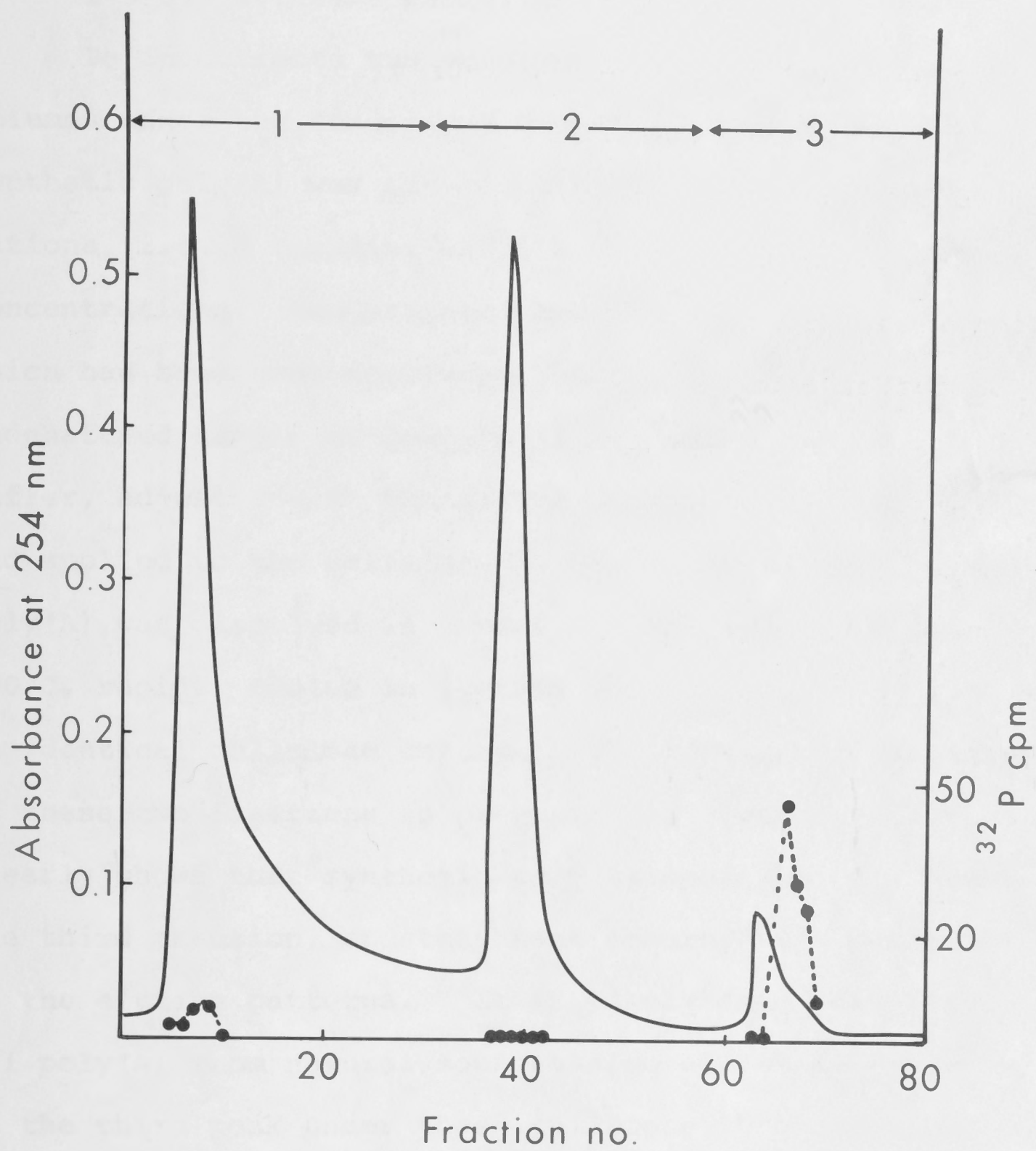
FIGURE 6-1    Cellulose column chromatography of ribonuclease  
resistant RNA isolated from  $^{32}\text{P}$ -labelled whole  
cell RNA

Animals had been injected intravenously with  $^{32}\text{P}$ -asorthophosphate, and sacrificed after 1 hour. The purified ribonuclease resistant RNA was dissolved in the first TSE:ethanol solution. 1, 2 and 3 represent elution with TSE:ethanol in the ratios of 65:35, 85:15 and TSE buffer alone respectively. RNA was applied to a 1.5 x 20 cm cellulose column and 3 ml fractions collected. 1 ml aliquots of fractions from each peak were separately dialyzed against a buffer containing 10 mM Tris-HCl, 1 mM  $\text{MgCl}_2$ , 0.5M KCl, pH 7.6, and these fractions assayed for radioactive  $^{32}\text{P}$  binding to millipore filters, as described.

\_\_\_\_\_ , Absorbance at 254 nm

●.....● , Radioactivity of filters.





total radioactivity of the poly(A) segments retained on all millipore filters summed to 13% of the total acid precipitable radioactive material, which included all the dsRNA and poly(A) eluting in the third peak fraction.

#### 6-3-3 *Cellulose Chromatography of Synthetic Poly(A)*

To investigate the behaviour of poly(A) on cellulose columns under the conditions normally used to prepare dsRNA, synthetic poly(A) was chromatographed under identical conditions, i.e. a stepwise elution with TSE:ethanol of differing concentrations. Two preparations of poly(A) were used, one which had been heat denatured and one which has not. The undenatured sample of poly(A) (2 mg) was dissolved in TSE buffer, adjusted with ethanol to a TSE:ethanol ratio of 65:35, and applied to the cellulose column. An identical amount of poly(A) was dissolved in 1 ml 0.1 x SSC and heat denatured at 100°C, rapidly cooled in ice and then similarly applied to an identical cellulose column. The results of chromatography of these two fractions is presented in Figure 6-2, which quite clearly shows that synthetic poly(A) segments only elute in the third fraction, and that heat denaturation has no effect on the elution patterns. It is likely from this result that all poly(A) from natural sources also elutes exclusively in the third peak under these cellulose chromatography conditions.

#### 6-3-4 *Oligo-(dT) cellulose Chromatography of Material eluted from the Third Peak of Cellulose Chromatography*

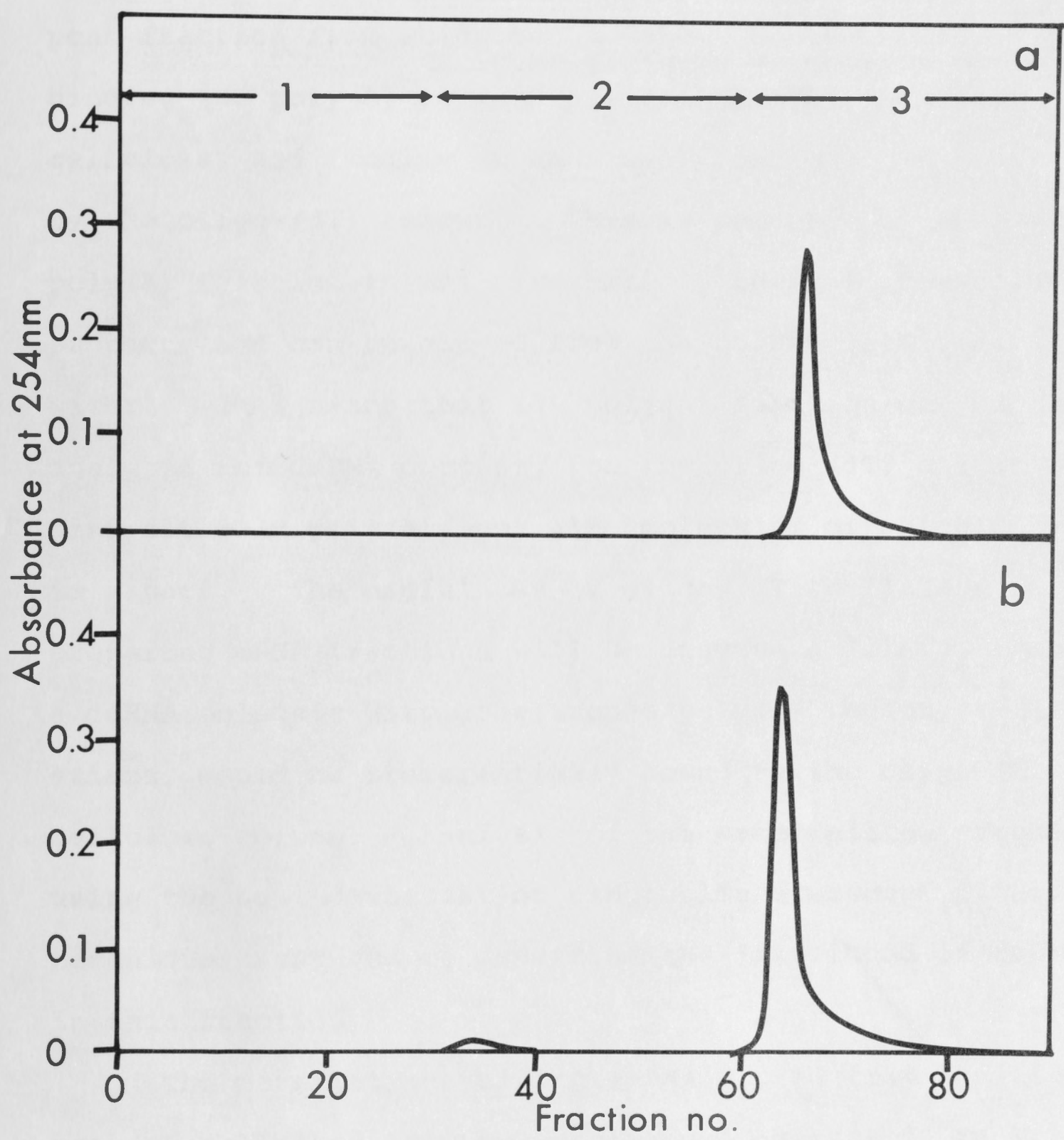
The millipore filter analysis, because it particularly

FIGURE 6-2-a and b    Cellulose column chromatography of  
(a) undenatured, (b) heat denatured  
preparations of synthetic poly(A)

Preparations of synthetic poly(A), one of which had been heat denatured in 0.1 x SSC, were dissolved in the first TSE:ethanol concentration. Column chromatographic elution is identical to that described in Figure 6-1. The poly(A) preparations were applied to identical 1.5 x 20 cm cellulose columns and monitored for absorbance at 254 nm.

\_\_\_\_\_, Absorbance at 254 nm





binds poly(A) in high KCl solution, is a useful tool for detecting poly(A), or removing it from RNA preparations. The aim of the experiments with oligo-(dT) cellulose chromatography was to firstly confirm the findings with millipore filters, secondly to examine more closely a third peak fraction from which the poly(A) had been removed by binding the poly(A) to the (dT) residues of the substituted cellulose, and finally to examine those residues retained by the oligo-(dT) column. This is possible because the poly(A) fraction is not irreversibly bound to the column support, and can be eluted from the column with distilled water. This means that the poly(A) fraction can be analyzed for dsRNA content, for the oligo-(dT) column will bind not only poly(A), but also molecules of which poly(A) is a part. The usefulness of oligo-(dT) cellulose in preparing mRNA fractions will be discussed later. Hence a dsRNA molecule with an attached poly(A) region, if it exists, would be preferentially bound to the oligo-(dT) cellulose column. Analysis of the water eluted fraction, using the heat denaturation ribonuclease susceptibility technique, was used to determine the likelihood of dsRNA in this fraction.

The material containing dsRNA eluted from the third peak of cellulose column was dialyzed against 20 mM Tris-HCl, 1 mM  $MgCl_2$ , and 0.5M NaCl, pH 7.4 and applied to a small column of oligo-(dT) cellulose, as described (6-2-2). The fraction passing through the column and the water eluted

TABLE 6-2

Analysis of the fractions either passing through or binding to oligo-(dT) cellulose after application of dsRNA third peak material eluted from cellulose. Material was applied (2832 cpm) in high salt buffer and eluted with distilled water (section 6-2-2).

	Elution volume (mls)	TCA precipitable (radioactivity) (total cpm of each fraction)
Fraction passing through oligo-(dT) cellulose without binding	1	2,300
	2	230
	3	-
	4	-
Total cpm of unbound fraction		2,530
Fraction bound and subsequently eluted with water	1	220
	2	100
	3	-
	4	-
Total cpm of eluted fraction		320
Eluted fraction of total applied		$\frac{320}{2850} = 11.2\%$



TABLE 6-2

fraction, were assayed for radioactivity and the results of this analysis are presented in Table 6-2. The result indicates that approximately 89% (2530 cpm) of the total radioactivity added, passed through the oligo-(dT) cellulose column, hence 11% was retained by the column but was eluted when washed with distilled water (320 cpm). The material not bound by the column is measured in the first and second 1 ml fractions and no further radioactivity could be measured after repeated washings with large volumes of the column buffer described above. The poly(A) fraction was immediately eluted within the first 2 ml of washing with distilled water. The total value of material collected from the column ( $2530 + 320 = 2850$  cpm) closely resembled the value determined independently initially for the radioactivity of the material to be applied, (2832 cpm) therefore no material remained on the column after the distilled water wash.

Both the distilled water wash and the material passing through the oligo-(dT) column, were assayed for dsRNA by the heat denaturation-ribonuclease susceptibility method. Both samples were dialyzed against low salt buffer (0.1 x SSC) and treated as indicated in Table 6-3.

A comparison of (1) and (2), i.e. the water eluted fraction after heat denaturation, but before (1) and after (2) ribonuclease treatment, indicate no difference in the radioactivity of this fraction. This clearly establishes

TABLE 6-3

Effect of heat denaturation and ribonuclease treatment on the fractions passing through or binding to oligo-(dT) cellulose. Fractions passing through or binding and eluted with water, were pooled and either heat denatured or heat denatured and ribonuclease treated, as indicated.

Fraction	Treatment	Acid precipitable radioactivity (cpm)
Bound	1. Heat denaturation only	184
	2. Heat denaturation followed by ribonuclease A (20 µg.ml), 37°C, 30 min.	187
Unbound	3. Heat denaturation only	924
	4. Heat denaturation followed by ribonuclease A (20 µg/ml), 37°C, 30 min.	153

6-3-5 Gel electrophoretic Analysis of the dsRNA

fraction before and after poly(A) Removal with

Oligo-(dT) Cellulose

These experiments were aimed at investigating the gel electrophoretic behaviour of the dsRNA material eluted from a cellulose column and to determine whether poly(A) removal affected the pattern. The description of gel electrophoresis

that the material bound is almost certainly all poly(A) segments, having no dsRNA material attached to them. In contrast, (3) and (4) indicate identical treatment of the material which passes through the column without binding. Heat denaturation, followed by ribonuclease treatment of this material, results in the loss of a large percentage of dsRNA due to separation of the strands during heat treatment.

The percentage of poly(A) fraction, i.e. the fraction bound to the oligo-(dT) cellulose column, was 11.2%. This result corroborates the observation of the millipore filter analysis, described above, wherein 13% of the dsRNA third peak fraction was retained by the millipore filters, and shows a favourable agreement with the figure of 12.5% excess adenylic acid obtained by base analysis. All these results therefore illustrate that poly(A) segments are present in the preparation of dsRNA, but constitute only a small percentage and are probably co-chromatographing in the third peak on cellulose. It therefore appears that poly(A) is present in the dsRNA preparation, but is not covalently linked to dsRNA.

6-3-5 Gel electrophoretic Analysis of the dsRNA fraction before and after poly(A) Removal with Oligo-(dT) Cellulose

These experiments were aimed at investigating the gel electrophoretic behaviour of the dsRNA material eluted from a cellulose column and to determine whether poly(A) removal affected the pattern. The description of gel electrophoresis

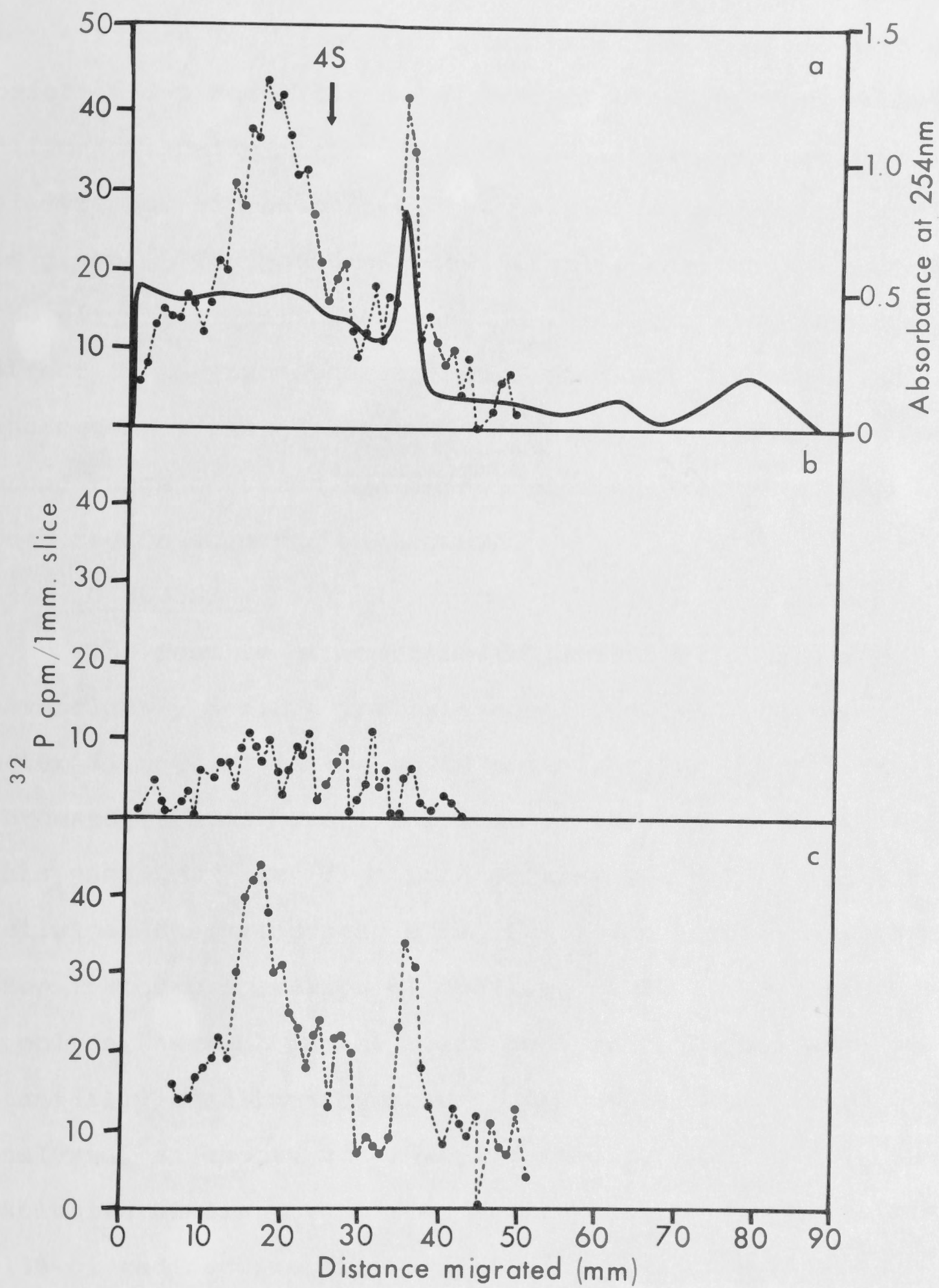


FIGURE 6-3-a, b and c    Migration of dsRNA isolated from  
 $^{32}$ P-labelled whole cell RNA in 10%  
polyacrylamide gels

The profile indicates the gel electrophoretogram of dsRNA before (a) and after (c) passage through an oligo-(dT) cellulose column to remove poly(A) segments. In addition the profile with water eluted fraction (b) is also given. Gels were scanned for absorbance at 254 nm and sliced into 1 mm slices and each assayed for radioactivity.

\_\_\_\_\_ , Absorbance at 254 nm

●.....● , Radioactivity/mm slice.



of dsRNA, and the subsequent finding of two different peaks with markedly different characteristics of homogeneity and specific radioactivity, is described in section (3-3-6).

Figure 6-3 illustrates the profiles observed with dsRNA before 6-3-a and after 6-3-c removal of poly(A) segments with oligo-(dT) cellulose. In addition, in 6-3-b, the gel electrophoretic pattern of the poly(A) water eluted fraction is given. The patterns show firstly, that the passage through the oligo-(dT) cellulose and the removal of poly(A), has no effect on the two observed peaks of dsRNA activity. The poly(A) subsequently eluted appears to be evenly distributed over a broad range of sizes and is not present exclusively in either peak region observed with dsRNA.

#### 6-4 DISCUSSION

The results of experiments presented in this chapter have clearly defined the existence of poly(A) segments in the material eluting in the third peak fraction of cellulose chromatography. The importance of this is obvious, because this same third peak fraction contains the dsRNA, and the cellulose chromatography constitutes the important purification step in the preparation of purified dsRNA. The existence of a poly(A) segment in the third peak fraction has been substantially confirmed by three independent methods: (1) base analysis, an excess of adenylic acid by 12.5%; (2) the exclusive binding of poly(A) segments to millipore filters, (13% of radioactive material bound); (3) the binding of poly(A) segments to oligo-(dT) cellulose (a fraction of 11.2%



bound to the oligo-(dT) cellulose which could then be eluted with water).

There is considerable agreement between the percentages obtained by these three independent methods. It is apparent therefore that between 11 and 13% of all material in the third peak of cellulose is poly(A).

This behaviour of poly(A) in co-chromatographing in the third peak region is also confirmed by the application of synthetic poly(A) to a similar column under identical conditions. Once again, the behaviour of synthetic poly(A) indicates that poly(A) elutes exclusively in the third peak after cellulose chromatography, and that this behaviour is not changed by heat denaturation of the original poly(A) applied. This result would appear to be inconsistent with that obtained by KRONENBERG and HUMPHREYS(1972) who are the only other workers to have investigated the possibility of poly(A) contamination of the third peak. These workers also used synthetic poly(A) under exactly the same conditions of cellulose chromatography, and reported that synthetic poly(A) chromatographed in the second peak on cellulose. Further investigation by these workers of a ribonuclease resistant fraction labelled for a longer time period revealed a high A content on base analysis of the fraction. In this material they obtained a 35% excess of A residues, hence suggesting a 35% contamination of dsRNA with poly(A). No other analysis of the dsRNA was conducted. However, such a high excess of A appears to be anomalous with their other result that synthetic poly(A) chromatographed in the second peak.

This anomaly might be in part explained by the differential capacity of cellulose to retain poly(A) (KITOS, SAXON and AMOS, 1972). Whatman CF-11 cellulose has a low capacity to retain poly(A) (DeLARCO and GUROFF, 1973; MERCER, 1974). Further studies have shown that the capacity to bind poly(A) appears to be due to polyaromatic impurities in the preparation of cellulose (SULLIVAN and ROBERTS, 1973) which may vary between batches of the same branch of cellulose and type of wash used in preparation (DELARCO and GUROFF, 1973; MERCER 1974).

In contrast, there appears to be only a 12% contamination with poly(A) in the dsRNA preparations from rat liver. Percentages higher than this value have never been obtained by any of the poly(A) analysis methods employed in this thesis. Concomitantly, synthetic poly(A) has been observed to chromatograph in the third peak on cellulose, and not the second.

The contamination with a small percentage of poly(A) with the dsRNA does not overall affect the properties which the dsRNA displays. It would be unlikely that when dsRNA constitutes the major proportion of the third peak fraction that there would be any effect on its heat denaturation, melting profile or immunodiffusion properties. Base analysis is affected but the excess due to poly(A) can be corrected. The gel electrophoretic profiles were examined to confirm the existence of two dsRNA species, and that neither of the observed peaks were in fact contained by poly(A) segments preferentially. There was no significant contribution to either peak.

The major question remaining was a possibility that dsRNA and poly(A) may be linked in some way, and that this may have an implication connected with the importance of poly(A) in the picture of post-transcriptional regulation. From the preliminary experiments revealed in this chapter, wherein the poly(A) fraction retained by an oligo-(dT) cellulose column was investigated for dsRNA behaviour, it would appear that the poly(A) and dsRNA are not covalently linked. No typical dsRNA behaviour, i.e. loss of ribonuclease resistance on heat denaturation, could be detected in the poly(A) retained fraction. It therefore seems that the poly(A) is merely co-chromatographing in the third peak fraction of cellulose chromatography.

However, it must also be realised that the situation between dsRNA regions and poly(A) "*in vivo*" may be different. Indeed the method of preparing dsRNA uses extensive digestions with ribonucleases and other purification methods. These techniques may cause a breakage in a bond involving dsRNA and poly(A). It is not unreasonable to suggest that in reality a bond may exist between dsRNA and poly(A), and that there may be an important function of dsRNA linked to that of poly(A). The finding that dsRNA probably arises in the main part from the looped regions of the HnRNA and the fact that a long poly(A) region is also present as part of this molecule (DARNELL *et al.* 1971; PHILIPSON *et al.*, 1971; MENDECKI, LEE and BRAWERMAN, 1972; and BIRMBOM, 1973) and may be involved in the processing of HnRNA into the mRNA of the cytoplasm, argues that dsRNA may also play a vital role in this processing of mRNA. This point



is developed more fully in the next chapter, which deals with the finding that specific hybridization is possible between an mRNA fraction isolated from polysomes and heat denatured dsRNA. Such hybridization also exists between heat denatured dsRNA and HnRNA molecules. The details of these experiments will be discussed in the following chapter.

and molecular localization. Following this is a discussion of the function of the dsRNA species. There are various aspects concerned with these likely functions, and they will be considered individually.

One particular aspect of function has been further investigated in some detail, but the work is incomplete. I will outline the experiments conducted in an attempt to illustrate that the functions of dsRNA are likely to be involved in the fundamental processes of cell machinery. This part of the work concerns the finding of specific hybridization of heat denatured dsRNA to an mRNA fraction and the HnRNA from which the mRNA was derived. While the discussion of Chapters V and VI leads on to this particular point, without placing undue emphasis on this role of dsRNA, it appears to be a particular property of dsRNA worthy of the present and continuing investigations. A larger part of this discussion will therefore deal with this involvement in cellular function. The aspects considered in this discussion will hopefully lead to the pursuance of further knowledge of the complete functions of dsRNA.

CHAPTER VII - DISCUSSION7-1 CHARACTERISTIC EVIDENCE FOR dsRNA

In this discussion I will firstly attempt to draw together the various aspects of the major part of this thesis, which has been concerned with the isolation and characterization of the dsRNA species, and its cellular and molecular localization. Following this is a discussion of the function of the dsRNA species. There are various aspects concerned with these likely functions, and they will be considered individually.

One particular aspect of function has been further investigated in some detail, but the work is incomplete. I will outline the experiments conducted in an attempt to illustrate that the functions of dsRNA are likely to be involved in the fundamental processes of cell machinery. This part of the work concerns the finding of specific hybridization of heat denatured dsRNA to an mRNA fraction and the HnRNA from which the mRNA was derived. While the discussion of Chapters V and VI leads on to this particular point, without placing undue emphasis on this role of dsRNA, it appears to be a particular property of dsRNA worthy of the present and continuing investigations. A larger part of this discussion will therefore deal with this involvement in cellular function. The aspects considered in this discussion will hopefully lead to the pursuance of further knowledge of the complete functions of dsRNA.

In Chapter II evidence was clearly presented for the occurrence of a dsRNA species in rat liver. Subsequently the presence of dsRNA was confirmed independently in rat testes. Although other organs, e.g. spleen and kidneys, were not investigated, there is a likelihood that dsRNA is universally present. In this thesis, however, the properties and characterization have been concerned with dsRNA from rat liver, but the implications of these findings will be extended to cover the properties and likely functions of dsRNA in general. If all the reports of ribonuclease resistant dsRNA species are considered, although there are many anomalies and inconsistencies in the reported values of dsRNA properties, which may be due to crude preparations, the wider occurrence of dsRNA in eukaryotic species seems evident. The functions of dsRNA are also likely to be similar in all eukaryotes.

Due to the complexity of the *in vivo* nature of dsRNA and the likelihood that it may be involved in many different roles in intact cells, each role must be explored separately, before a final conclusion can be reached as to the complete function of dsRNA(s). Our first priority, in pursuing a study of dsRNA, was to establish conditions of preparation, purification and identification which could be applied not only to the dsRNA species in rat liver, but also to future studies of similar species of other eukaryotic sources. The studies outlined in Chapters II and III were designed with this goal in mind, and the investigations reveal several points. The results indicate that ~~dsRNA can be only practically~~



~~prepared by the removal of single stranded RNA fragments from the dsRNA using the two specific ribonucleases, but~~ that rigorous attention must be given to the ribonuclease digestion conditions and concentration. In the reports of dsRNA published since the experiments in this thesis were begun, there has been a consistent failure to understand the importance of establishing the optimum ribonuclease:RNA ratio, which is essential for single stranded RNA removal, but does not allow any breakdown of the double stranded structure. The conditions of ribonuclease treatment and purification methods by other workers were given in detail in Table 2-5, which lists all the references to recent reports of dsRNA from similar "uninfected" sources. The tables are discussed in greater detail in Chapter III, but it is worth mentioning that aside from the inconsistencies in the actual ribonuclease concentrations and digestion conditions, or the lack of ribonuclease  $T_1$ , in no report was any reference made to the concentration of RNA, or the ribonuclease:RNA ratio (see Table 2-5 for references). Failure to define these conditions may lead to erroneous interpretations of results of experiments conducted with impure or partially digested material. For example, a high content of dsRNA as part of the HnRNA (3%) was observed by JELINEK and DARNELL, (1972). This high content of dsRNA is likely to be due to the insufficient ribonuclease digestion of the original material. The importance of this will be illustrated in later discussions of the HnRNA-mRNA theory. The conditions required for

successful removal of single stranded fragments from rat liver cellular RNA were carefully established in the first chapter, and these conditions could be adopted for any cellular RNA species from other sources.

Another important consideration is the purification of dsRNA, for although ribonuclease resistant preparations could be purified to remove any attached protein or DNA, normal methods, e.g. ethanol precipitation, did not necessarily remove small single stranded RNA fragments remaining. Considerable weight was given in the discussion of chromatographic separation (Chapter II) to the importance of the point that the method of purification should be determined by ~~its~~RNA separation based on its actual base-paired structure, rather than its molecular weight. Molecular weight sieve techniques were used as purification methods by KIMBALL and DUESBERG, (1971); HAREL and MONTAGNIER, (1972) and RYSKOV *et al.*, (1973). In each case the preferred method was either Sephadex or agarose separation. However, molecular weight separation may be misleading, as the technique does not clearly establish that a fraction eluting at a particular elution volume is a double stranded molecule entirely, or a double stranded molecule with a single stranded tail. The two species may have the same molecular weight and hence behave similarly on a molecular weight sieve, but in reality they may be different species. It is not possible to say in each case with the results of KIMBALL and DUESBERG, (1971); HAREL and MONTAGNIER, (1972) or RYSKOV *et al.*, (1973) whether

this method of purification results in erroneous estimations, but one property which is likely to be significantly affected is the base analysis of the purified material. The diversity of purification techniques used may explain the variability in the base analysis results.

In this thesis, two purification methods were used. The Sephadex separation method was found to be unsatisfactory largely for the reason described above. It is important that estimations of dsRNA character, particularly base analysis, should not include single stranded RNA tails. To a large extent the cellulose chromatographic technique can eliminate the difficulties encountered with molecular weight sieve methods, for although the actual mechanism of separation is not understood, the method is known to achieve the separation by differences in the conformation of the molecules, eliminating the possibility of fractionating short dsRNA regions with long single stranded tails. The hydroxyapatite method can be used for purification, and was actually used by JELINEK and DARNELL, (1972). This method does not appear to be as reliable as cellulose in eliminating the above difficulty. KRONENBERG and HUMPHREYS, (1972) are the only others reported to have used the cellulose purification technique.

The cellulose method has other advantages. One of these is that it can be used to prepare large quantities of dsRNA which is a very small proportion of the total extractable RNA. Without exploiting this particular property, experiments requiring large quantities of dsRNA would not



have been possible. Restriction on the amount of dsRNA which could be prepared is probably a major reason why other workers have been unable to further their investigations of the ribonuclease resistant species.

Early confirmation that the cellulose technique was suitable for the preparation of purified dsRNA was obtained primarily from the observation that naturally occurring dsRNA molecules from reovirus, and synthetic dsRNA molecules, poly(I).poly(C) chromatograph solely in the third peak region. Since KRONENBERG and HUMPHREYS, (1972) are the only other workers to have employed this technique, the findings associated with the cellulose chromatographic method are not easily compared. These workers do report dsRNA from sea urchin embryos in the third peak, but interpretation of some of their results is difficult. For instance, they find that the poly(A) material chromatographs in the second peak. A suggestion to explain this anomaly was made in Chapter VI, but the base analysis of the third peak appeared to reflect a high A value, despite the assurance of no poly(A) in their third peak material. Poly(A) is present in rat liver purified dsRNA, but correction can be made to allow for this observation, or it can be successfully removed with oligo-(dT) cellulose if necessary. One other noticeable feature of the cellulose separation technique is that dsRNA chromatographs only in the third peak; this feature can be used as a dsRNA identification method.

Further confirmation of the double stranded nature has been repeatedly obtained by various investigations using identifying properties peculiar to dsRNA and not single stranded RNA. The investigation of the material purified by cellulose chromatography appears to fulfill all the known properties distinguishing dsRNA, and is thus a further reason for the suitability of the cellulose technique.

The properties of dsRNA which are peculiar to dsRNA were outlined in the Introduction, but they include:

(1) ribonuclease resistance; (2) differential solubility; (3) heat denaturation: characteristic biphasic profile; (4) base analysis:  $A = U$ ,  $G = C$ ; (5) reaction to specific antigens<sup>sera</sup>; (6) specific behaviour on chromatography, e.g. cellulose or hydroxyapatite. In the experiments detailed in this thesis, each of these properties, except the differential solubility which was observed but no detailed investigations of this property were conducted, was rigorously examined. The results indicated in every case that the purified presumed double stranded material from rat liver behaved as dsRNA. In contrast, such a comprehensive examination of the purified material has not been carried out by any other group of investigators. The findings in this thesis will be detailed briefly. The material exhibited:

(1) Ribonuclease resistance: the material was prepared by this method and showed further ribonuclease resistance typical of dsRNA.

(2) Heat denaturation profile: A biphasic heat denaturation profile, with a sharp transition at a defined melting temperature ( $T_m$ ) from ribonuclease resistance to ribonuclease sensitivity.  $T_m$  depends on salt concentration.

(3) Base analysis: The base analysis figures show  $A = U$ , and  $G = C$ , when corrected for poly(A) content. The  $G + C$  value also indicates the identical  $T_m$  to that observed in the heat denaturation profile, according to the linear relationship of  $G + C$  to  $T_m$  values.

(4) Caesium sulphate density gradient profile: A density profile was obtained intermediate between single stranded RNA and DNA as would be expected of dsRNA. The density is similar to naturally occurring dsRNA from reovirus.

(5) Reaction to specific antigens<sup>Sera</sup>: Both dsRNA from rat liver and testes showed immunospecific reaction to an anti-serum specifically prepared against synthetic poly(I).poly(C). This antiserum did not react to single stranded RNA, or double stranded DNA, or dsRNA which was heat denatured.

(6) Chromatographic behaviour: The behaviour of dsRNA in chromatographing in the third peak on cellulose.

Obviously this is not the first or only study to attempt to characterise dsRNA using these properties. Throughout the thesis reference has been made to other investigators who have attempted to isolate, purify and characterize material as dsRNA. In each case the characterization was done by taking their material and examining it



for one or more of the known dsRNA properties. Where possible in this thesis, an attempt has been made to draw comparisons with other recent findings concerning "~~uninfected~~" dsRNA from <sup>uninfected cells,</sup> ~~other sources~~. An illustration of the dsRNA properties which were examined to characterize material as dsRNA by other workers is given in Figure 7-1. In each case if the property was examined and appears to show dsRNA behaviour, it is indicated by a +, if negative behaviour a -, and o for not examined, and ± where the property was examined but the behaviour was not obviously indicative of dsRNA. This method only allows us to summarize the characterization methods, but does not illustrate subtle differences in the heat denaturation temperature etc. Observed differences in base analysis, melting temperature, etc. may be mainly due to the diversity of the preparation or purification methods, e.g. RYSKOV *et al.*, (1973) used a hot-phenol differential extraction technique for preparing nuclear RNA from whole cells, whereas in experiments both by JELINEK and DARNELL, (1972) and in the experiments described in this thesis, the nuclei were prepared first and then extracted. Similarly no account has been taken of the very different ribonuclease conditions and concentrations which prevail (see Table 2-5).

A brief summary of the findings of other workers illustrates a few of the differences between dsRNA characterizations observed. All material isolated by all workers was ribonuclease resistant due to the fact that all were isolated from whole RNA by ribonuclease digestion.

TABLE 7-1

Author and Date	Source (not all sources were investigated for each individual property)	RNase Resistance	Heat Denaturation	Base Analysis	Reaction to Specific Antigens <small>Sera</small>	Caesium Sulphate	Characteristic Chromatography
Stein and Friedman 1970, 1971	Burkitts lymphoma Human lymphocytes CEF	+	+	+	0	0	0
Kimball and Duesberg, 1971	Rabbit kidney CEF Hela	+	+	0	0	+	0
Harel and Montagnier, 1972	Rat liver	+	+	+	0	0	0
Kronenberg and Humphreys, 1972	Sea urchin embryo	+	+	+	0	0	+
Jelinek and Darnell, 1972	Hela cell nuclei	+	0	+	0	+	+
Rskov <i>et al.</i> , 1973	Erhlich ascites	+	+	+	0	0	0
Patnaik and Taylor, 1973	Erhlich ascites sarcoma	+	+	0	0	0	0
Monckton, 1974	1. Whole cells 2. Cytoplasm 3. Nuclei	+	+	+	+	+	+

STERN and FRIEDMAN, (1970, 1971) examined two other dsRNA characteristic properties in material isolated from Burkitt's lymphoma. They discovered a  $T_m$  of  $86^{\circ}\text{C}$ , but 60% of their material remained resistant after heat denaturation. Their base analysis on material from embryo fibroblast cells revealed equal A, U, and C values but a high G value. The  $T_m$  observed in their heat denaturation profile does not fit the linear correlation between  $T_m$  and G + C content described in Chapter III.

KIMBALL and DUESBERG, (1971) examined the heat denaturation profile in all three cell types and obtained a  $T_m$  of  $86^{\circ}\text{C}$  in low salt, these high values are more indicative of a viral origin for the dsRNA. They also examined the caesium sulphate density gradient centrifugation pattern and obtained profiles indicative of dsRNA lying between single stranded RNA and DNA, however this property does not exclude the possibility of a viral origin.

HAREL and MONTAGNIER, (1972) have shown a heat denaturation profile with a transition in ribonuclease sensitivity at a  $T_m$  of between  $75$  and  $77^{\circ}\text{C}$ . In earlier studies (MONTAGNIER, 1968) base analysis revealed similar G and C values (22.3 and 20.5 respectively) but a high A value (30.5). The significance of poly(A) was not realised in 1968 when this analysis was conducted. Several criticisms of this work have been made elsewhere in this thesis, in particular the lack of ribonuclease  $T_1$  in the digestion step and inadequate purification techniques. No chromatographic examination was made to identify their material.



KRONENBERG and HUMPHREYS, (1972) showed a heat denaturation temperature of 76°C, and a cellulose profile characteristic of dsRNA. The base analysis of the third peak material indicated approximately equal values for U, G and C, but the A value was high. It appears that the ribonuclease resistant material isolated was contaminated with poly(A) segments. However they observed poly(A) chromatographing in the second peak on cellulose. This point was discussed in detail in Chapter VI, but it is likely that the high value for A in base analysis reflects contamination with poly(A) segments. Generally the analysis for dsRNA character was fairly thoroughly investigated.

The work of JELINEK and DARNELL, (1972) has indicated some important functions for dsRNA and, in particular, an indication of the derivation of dsRNA from the HnRNA. Nevertheless, they did not examine the heat denaturation profile, a most important dsRNA character, but instead based their dsRNA characterization on the caesium sulphate density gradient behaviour, where they observed patterns with the same density value as reovirus dsRNA. The purification step used hydroxyapatite, which may be indicative of dsRNA character and the base analysis revealed equal A and U values, but high G and C. However, it does not exclude the possibility of contamination with RNA in single stranded tails associated with dsRNA. These high G and C values are probably indicative of the lack of

ribonuclease  $T_1$  (see later part of Discussion).

RYSKOV *et al.*, (1973) have based their dsRNA premise on the basis of its heat denaturation behaviour on hydroxyapatite but no heat denaturation temperature was given. They revealed the presence of two dsRNA species, the larger of which appeared to contain high A residues, and the smaller of which seemed to be distinctly G, C rich. PATNAIK and TAYLOR, (1973) examined the heat denaturation behaviour to identify the double stranded nature of their material isolated from ascites cells. No other examination for dsRNA characteristic was carried out. The thermal denaturation behaviour was measured by a hyperchromicity method and a  $T_m$  of 64.39 obtained. This value was obtained with ribonuclease resistant RNA from sarcoma 180 cells.

Generally it can be seen that other workers have relied on the ribonuclease resistant behaviour to define the double stranded nature of their material, and in some cases examined one or two other properties. Each report in its own way contributes significantly to the understanding of dsRNA, but too often the basic properties defining a dsRNA have not been examined. It seems, therefore, that the investigations of dsRNA character by other workers has not always been satisfactory, and does not allow confidence in a final decision that the dsRNA is not contaminated with single stranded RNA fragments.

From this brief illustration of the deficiencies and discrepancies in the work on other dsRNA species, it is

obvious that the work on dsRNA in rat tissues and rat liver in particular, was conducted so that all the properties indicative of dsRNA were examined. The material examined fulfilled all those requirements, and also recommends the cellulose technique as the method for preparing pure intact dsRNA. Subsequent properties of purified dsRNA are then undistorted by single stranded RNA contaminants.

It was not the intention of this part of the discussion to repeat conclusions dealt with in detail at the end of each chapter. A restatement of the various properties which are the vital indicators of the double stranded nature was made to emphasize three particular points. In any study where the involvement of a dsRNA sequence is suspected, an experiment should be carried out; (1) under the proper conditions of preparation; (2) using adequate purification to remove all single stranded RNA, and (3) the resulting purified material should exhibit the maximum variety of unique dsRNA properties. If these requirements are not fulfilled, subsequent biological functions may be misinterpreted.

## 7-2 ORIGINS: CELLULAR AND MOLECULAR

The second part of this thesis was concerned with the cellular and molecular origin of dsRNA, and additional features such as the coexistence of poly(A) segments in dsRNA preparation isolated by cellulose chromatography, are also considered. The question of origin and subsequent finding of substantial evidence for HnRNA as the source of



the majority, if not all of dsRNA in the cell, leads to the question originally posed; What is its function? Before discussing this aspect, the findings <sup>on the</sup> ~~of~~ origin will be briefly reviewed.

Conclusive evidence was obtained for the existence of dsRNA within the nucleus. dsRNA isolated from rat liver nuclei has the same properties as dsRNA isolated from the whole cell. The evidence concerning nuclear dsRNA is summarized as follows:

- (1) dsRNA prepared from isolated nuclei of rat liver cells has a profile on cellulose similar to that obtained from whole cells. The profile shows the third peak characteristic of dsRNA.
- (2) The third peak on cellulose chromatography represents a higher proportion of the total extractable RNA than is isolated for whole cells, suggesting a nuclear origin.
- (3) The third peak fraction has identical physico-chemical properties to whole cell dsRNA. In particular, both dsRNA of cellular and nuclear origin showed the same  $T_m$  value, 94°C in high salt solution.
- (4) The amount of third peak material on cellulose chromatography of ribonuclease treated cytoplasmic RNA was negligible (0.03% of whole RNA in the cytoplasm). There is a possibility that dsRNA was not detected by the method outlined in the present experiments, and a small fraction may be found associated with individual cytoplasmic organelles.

(5) dsRNA was detected in an unreduced amount in whole rat liver cells, labelled with [ $^3\text{H}$ ] orotic acid and extracted after 20 min. Under these labelling conditions, very little labelled RNA was detected in the cytoplasm other than terminal addition to tRNA. It appeared that dsRNA was as rapidly labelled as HnRNA and pre-mRNA fractions.

(6) Similarly an unreduced amount of dsRNA was detected in preparations of RNA where the synthesis of ribosomal precursors and ribosomal RNA was suppressed by low levels of actinomycin D. Possible dsRNA regions derived from pre-rRNA or rRNA did not, therefore, contribute to the dsRNA isolated.

(7) tRNA was not a source of dsRNA, having only small base-paired regions, and the possibility of a DNA-RNA hybrid was eliminated by the finding that material from the third cellulose peak, labelled for both DNA and RNA, was completely susceptible to alkaline hydrolysis.

Collectively, the evidence indicated HnRNA as the likely source of the majority, if not all of the dsRNA, within the cell. It appeared to derive from the "hairpin" regions of the HnRNA molecule.

The nuclear origin was investigated more closely, in particular the nuclear RNA species were fractionated into different molecular weight subclasses. dsRNA was found in each of these molecular weight species. The "snap-back" technique was used to analyze each of the subclasses as it

appeared that all of the dsRNA recovered from high molecular weight HnRNA was derived from loops. The gel electrophoretic profile established that the size of dsRNA was not homogeneous, but the average size of the larger dsRNA species appears to be 60 nucleotide pairs. Interpretation of the analysis of the other subclasses is a little more complicated where the values indicate that between 15 and 50% from subclass I to IV respectively, may be derived from "hairpin" loops. The lower recovery may be due to the presence of nicked or degraded RNA, or may be due to the presence of true inter-molecular <sup>double-stranded</sup> forms of RNA. It is not possible from the present investigation to detect which of these possibilities is the case, and it is possible that the true picture may reveal that all dsRNA is derived from loops. Further investigation is required to determine the significance of dsRNA in low molecular weight nRNA. Nevertheless, there is a possibility that a true independent form of dsRNA exists in the cell. The size of this species would be expected to be in the 4S region and may in fact be the smaller species observed on gel electrophoretograms. Only further analysis would reveal the significance of the smaller homogeneous species of dsRNA.

The importance of dsRNA in normal cell processes will be discussed in the remaining part of this chapter. These discussions will involve the functions of dsRNA, whether it is derived from HnRNA or of another origin. The significance of an independent molecular species of dsRNA will be



dealt with in considering the integrated observations made on dsRNA from uninfected rat tissues when compared with other dsRNA observations.

### 7-3 FUNCTIONS OF dsRNA

The functions of dsRNA are likely to be as diverse as its origins. However, the investigations have led to the finding that the major proportion of dsRNA synthesized in the cell, probably arises from the "hairpin-like" regions of the HnRNA. The following section will outline the extended studies which have been carried out on purified dsRNA, and in particular the hybridization studies with other species of RNA to determine the likely function of dsRNA. The hybridization studies will also be discussed in relation to the theory of mRNA processing from HnRNA, and functions of HnRNA in which dsRNA is likely to be involved.

The latter part of this discussion will deal with other possible functions of dsRNA. These include dsRNA involvement in interferon production and protein synthesis. The dsRNA studies in these two areas are quite diverse, and extensive theories have been suggested for dsRNA action on polysome accumulation, initiation factors and mRNA translation. Studies on interferon production are not quite so extensive and at first there may appear to be distinct contradictions in the mode of action proposed for dsRNA. It must be realized that each separate function proposed for dsRNA was investigated by different research groups, and in entirely different ways. An attempt will be made to bring together ideas on dsRNA function where possible, but it must be also remembered that more than one species of dsRNA may account

for more than one function.

### 7-3-1 *dsRNA Involvement in the Relationship between HnRNA and Cytoplasmic mRNA*

In view of the finding that dsRNA was derived from loops of the HnRNA in the nucleus, the question was then asked as to the possible function or involvement of a dsRNA structure in a fundamental genetic molecule such as HnRNA. Attention has been focused on HnRNA in particular, because it appears to be the genetic sequence from which mRNA may be processed or derived. It was suspected that dsRNA may play some part in this process of mRNA production.

It must be mentioned before briefly summarizing the evidence for mRNA being processed from the HnRNA sequence, that conclusive proof that a precursor-product relationship does not exist, nevertheless, recent findings have shown substantial evidence for this relationship. A discussion of the background of the HnRNA-mRNA relationship is necessary to understand the importance of the function of dsRNA.

Evidence for the relationship is obtained from several sources: (1) HnRNA has been shown to have a base composition similar to that of DNA (ATTARDI *et al.*, 1966; SCHERRER *et al.*, 1966) and which is very different from rRNA. (2) Hybridization and competition experiments of HnRNA and mRNA to DNA have demonstrated that only some sequences in HnRNA are similar to some sequences in mRNA, but are not necessarily identical (BIRNBOIM, PENE and DARNELL, 1967; SOEIRO and DARNELL, 1970; SCHERRER *et al.*, 1970; GREENBERG and PERRY, 1971) and in addition that some regions of HnRNA are probably

not represented at all in mRNA (SHEARER and MACARTHY, 1967; GEORGIEV, 1969; SOEIRO and DARNELL, 1970). (3) An attempt has been made to establish this relationship using RNA-RNA or RNA-DNA hybridization. An anti-mRNA complementary to 10S haemoglobin mRNA from anaemic duck erythrocytes was prepared. 10% of the heavier and 20% of the lighter fractions of HnRNA also prepared from duck erythrocytes, hybridized to the anti-mRNA (MELLI and PEMBERTON, 1972; IMAIZUMI, DIGGLEMAN and SCHERRER, 1973). (4) Low doses of actinomycin D, although completely inhibiting rRNA synthesis, produce virtually no effect on the synthesis of HnRNA or cytoplasmic mRNA. However, the synthesis of both HnRNA and cytoplasmic mRNA is completely suppressed by high doses of actinomycin D. (PENMAN *et al.*, 1968; PERRY and KELLEY, 1970). (5) Transformed cells containing viral DNA (SV40) covalently integrated into the cell DNA (SAMBROOK *et al.*, 1968) were shown to produce RNA molecules of  $1-5 \times 10^6$  daltons containing virus specific regions (LINDBERG and DARNELL, 1970; TONEGAWA *et al.*, 1970; WALL *et al.*, 1973) while the virus specific mRNA in such cells was discrete and of lower molecular weight (WALL *et al.*, 1973). During lytic infection with some DNA viruses, very high molecular weight virus-specific nuclear RNA was observed, while it was generally found that virus mRNA which was obtained from cytoplasmic polyribosomes was smaller (ROIZMAN *et al.*, 1970; PARSONS and GREEN, 1971). (6) Selective recognition of RNA by ribosomes associated with binding factors has been observed with whole nRNA,



tissue specific HnRNA, and cytoplasmic mRNA (NAORA, and KODAIRA, 1968, 1969, 1970). In addition, it appears that the protein factors associated with ribosomes are responsible for this recognition (NAORA, KODAIRA and PRITCHARD, 1971; NAORA and PRITCHARD, 1971; WHITELAM and NAORA, 1972). The specific recognition of HnRNA and mRNA by ribosomes suggest that HnRNA contains specific ribosome binding sites which may be similar to those of mRNA.

(7) Recent evidence has indicated that existence of a sequence of poly(A) in both HnRNA and mRNA (LEE, MENDECKI and BRAWERMAN, 1971; DARNELL, WALL and TUSHINSKI, 1971; SHELDON, JURALE and KATES, 1972) which appears to be about 200 nucleotides long (MENDECKI, LEE and BRAWERMAN, 1972; SHIENESS and DARNELL, 1973) and is located in both HnRNA molecules and mRNA molecules exclusively at the 3'OH terminus (MENDECKI, LEE and BRAWERMAN, 1972; MOLLOY and DARNELL, 1973). This finding in itself is evidence for the derivation of mRNA from HnRNA, but additional evidence has shown that the addition of 3'deoxyadenosine (cordycepin) can cause a blockage of the addition of poly(A) to HnRNA (MENDECKI, LEE and BRAWERMAN, 1972; JELINEK *et al.*, 1973) which then results in a failure of the appearance of newly formed mRNA in the cell cytoplasm (PENMAN, ROSBACH and PENMAN, 1970; ADESNIK *et al.*, 1972). (8) Direct evidence for the existence of mRNA sequences in HnRNA was obtained by analysis of the *in vitro* products translated from isolated HnRNA. A large HnRNA molecule prepared from duck

techniques of extraction, however, the gel pattern showed a length for HnRNA of approximately 50 base pairs. Within the

reticulocytes, even after centrifugation through a gradient containing 99% dimethyl sulphoxide, contains globin mRNA sequences which have been assayed in a cell-free protein synthesizing system (RUIZ-CARRILLO *et al.*, 1973). Heavy chain immunoglobulin or globin synthesis was found when HnRNA prepared from myeloma or mouse foetal liver cells was injected into *Xenopus* oocytes (STEVENS and WILLIAMSON, 1973 and WILLIAMSON *et al.*, 1973).

The evidence cited in all eight sections above is overwhelmingly in favour of HnRNA being the precursor to mRNA, and in particular the most recent findings in 7 by DARNELL and his co-workers. The significance of dsRNA in this theory does not at first seem evident, however dsRNA does appear to be a part of the HnRNA long molecule and therefore may be involved in this mRNA processing or indirectly involved in another way.

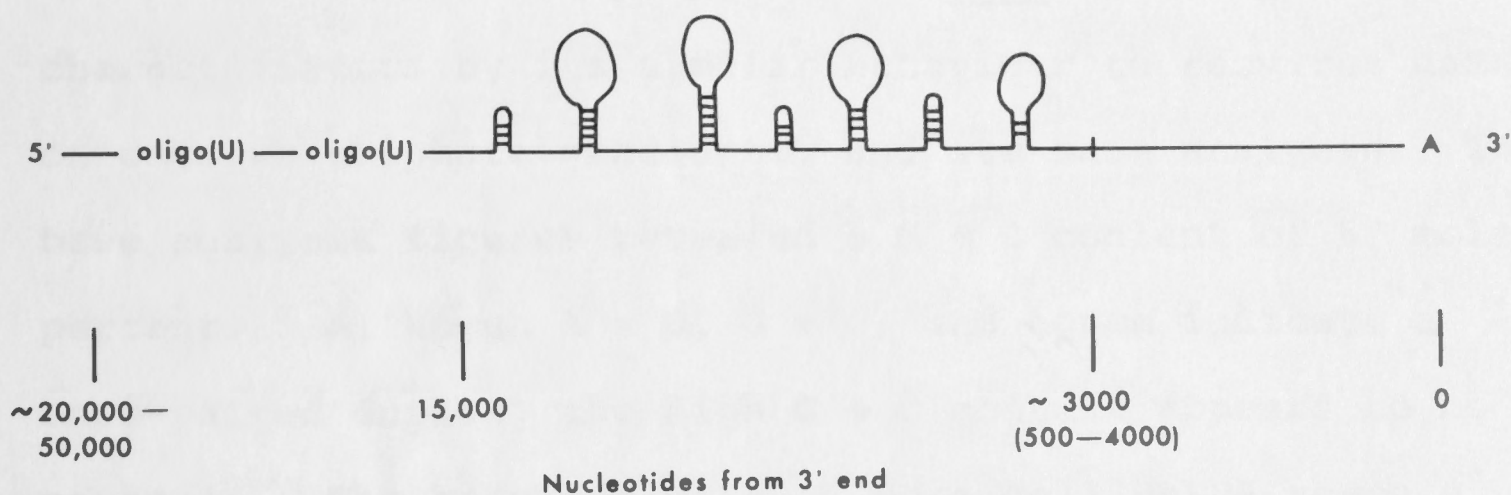
The importance of the dsRNA involvement becomes obvious when the actual processing of HnRNA is considered. The "snap-back" experiments reveal that the dsRNA apparently derived from "hairpin" loops in the HnRNA molecule constitutes 0.45% of the high molecular weight HnRNA. If one accepts the size of the high molecular weight HnRNA molecule sedimenting in the 40-70S region as being 10,000-20,000 nucleotides long (JELINEK and DARNELL, 1972) then the size of the section of the HnRNA molecule likely to be in a hairpin loop would be between 45 and 90 nucleotides long. It is difficult to absolutely exclude degradation of the RNA, or losses due to techniques of extraction, however, the gel pattern showed <sup>the</sup> length for dsRNA ~~of approximately~~ <sup>to be in the order of</sup> 60 base pairs. Within the

limits of some experimental error in the value of 0.45%, it would appear likely that the number of "hairpin" regions or dsRNA regions, is only one per length of HnRNA molecule. JELINEK and DARNELL, (1972) have reported the percentage of dsRNA of nuclear RNA to be 3%. Using the figure of 10,000 to 20,000 nucleotides for the length of HnRNA, this makes their estimate of the bases involved in sequences that are double stranded 300-600 nucleotides. JELINEK and DARNELL, (1972) did not predict the number of base paired regions, on the basis that their 15% acrylamide gel profile revealed a rather heterogeneous pattern and hence they did not deduce the exact size. Nevertheless, MOLLOY and DARNELL, (1974) and DARNELL, (1974) indicated a model of HnRNA showing several base paired regions (see model reproduced in Figure 7-1) in HnRNA.

In a report by RYSKOV *et al.*, (1973), they indicated the proportion of dsRNA to be between 0.8 and 1.2% of the high molecular weight HnRNA, and stated that they never observed a percentage as high as 3% as given by JELINEK and DARNELL, (1972). This would mean that RYSKOV and co-workers' reported dsRNA would consist of between 80 and 240 nucleotides in the HnRNA, representing one or two regions of dsRNA "hairpins", if the dsRNA observed by RYSKOV *et al.*, (1973) was the same size as rat liver dsRNA.

The value of 0.45% given in this thesis, would appear to be a reliable estimate, since dsRNA was prepared under ribonuclease digestion conditions, with isolation and





**Fig. 7.1. Model of HnRNA Molecule**  
 The loops and stems indicate regions of secondary (presumably base paired) structure  
 (from Molloy et al. 1974 Cell 1, 43. )

purification procedures carefully chosen for this purpose. The discrepancy between this low value and the 3% value observed by JELINEK and DARNELL, (1972) may be explained if one considers that the percentage of dsRNA obtained by these workers was deduced from experiments in which extremely mild ribonuclease digestion conditions and unsatisfactory purification procedures were used to prepare the dsRNA. The dsRNA prepared was only analyzed for dsRNA characteristics by its similar behaviour to reovirus dsRNA on caesium sulphate gradients, and its base analysis. The base analysis figures revealed a G + C content of 57 moles percent. Although  $A = U$ ,  $G = C$ , and hence indicate a base-paired duplex, the high G + C content appears to be unusual. The base analysis of Hela cell HnRNA reveals low G + C content (44 moles %). In contrast, the G + C content of precursor rRNA is high (70 moles %) (DARNELL, 1968). Unless an assumption is made that dsRNA regions, as part of HnRNA, are particularly G + C rich regions, a high value for dsRNA is difficult to explain. Alternatively the high G + C content may have been due to contamination of rRNA and/or precursor rRNA fragments, which resulted from inappropriate ribonuclease treatment and an inadequate purification method. The base analysis of rat liver dsRNA reveals a G + C content of 43 moles % (see Chapter III), which is not unlike the expected low G + C content of rat liver HnRNA. As a result of the inadequate attention paid to ribonuclease digestion and the anomalous base analysis figures given by JELINEK and DARNELL, (1972), it is likely

that 3% is an overestimate of the dsRNA content of HnRNA. Summing up all of these observations it appears likely that only one dsRNA region exists in HnRNA.

The question of the actual form of the dsRNA loop in HnRNA is also unexplained. In the model suggested by DARNELL, (1974) he proposes that the loops connecting the two halves of the dsRNA sequence may be quite large (see model in Figure 7-1). These larger connecting loops might be expected to be ribonuclease sensitive. Thus the larger looped "hairpins" would actually behave as inter-molecular bonded structures. JELINEK and DARNELL, (1972) only achieved 70% recovery in their "snap-back" analysis of high molecular weight HnRNA. The ribonuclease conditions for digestion of the pre-treated and untreated HnRNA were also very mild (2  $\mu$ g/ml ribonuclease A, 37°C, 1 min). In contrast, in the present experiments 100% recovery was obtained with more adequate ribonuclease digestion (37°C, 30 min), after previous heat denaturation. One interpretation of their failure to find 100% recovery would be that a "hairpin" may have a large inter-connecting loop, so that a perfect sequence match would not occur during rapid cooling. Alternatively, their HnRNA may have contained artificial nicks in the dsRNA region which occurred during the isolation of HnRNA. On the other hand, 100% recovery detailed in this thesis, indicates that the "hairpin" is a fully closed structure with no large loop. The "hairpin" would then appear almost as a 'palindrome' viz, a structure in which a sequence of bases is followed



immediately by a reverse repeat of the same sequence, allowing a close "hairpin" fold. The subject of 'palindromes' in RNA and DNA sequences is still under investigation. The extent of mismatching of bases in the dsRNA region is unknown. No absolute decision can be made on whether dsRNA occurs as a true 'palindrome' in the HnRNA from rat liver, but it is likely to exist as a close "hairpin" fold. Interesting observations have been made with mRNA sequences in which it has been predicted from the sequences of amino acids in the polypeptides coded for by RNA molecules, that close "hairpins" may exist (STEITZ, 1969; CORY *et al.*, 1970; JEPPESEN *et al.*, 1970; LAUX, DENNIS and WHITE, 1973).

The other involvement of dsRNA in the HnRNA-mRNA precursor relationship is concerned with a recent finding in our laboratories that hybridization has been demonstrated between preparations of rat liver mRNA and dsRNA prepared as described in this thesis (NAORA *et al.*, 1974). These hybridization studies are still incomplete and consequently the detailed aspects have not been included in this thesis. Specific hybrids have been demonstrated between preparations of heat-denatured dsRNA and mRNA. These hybrids were not the result of non specific aggregation of RNA nor were they due to hybridization with contaminants of either preparation with nuclear RNA. Specific hybrids have also been shown between dsRNA and nRNA, but not tRNA, rRNA or mRNA from heterologous cell sources, such as sarcoma 180 cells.

Characterization of the mRNA preparation used was carefully carried out. The results indicate that the preparation was certainly mRNA and was free from DNA. dsRNA-mRNA hybrids have been shown to form with low and high thermal stabilities.

The finding of specific hybrids between both nRNA and mRNA is in accord with the evidence illustrating that mRNA is derived from HnRNA. Indeed the finding of specific sequences in both mRNA and nRNA which are similar or identical to the dsRNA would appear to be in accord with the HnRNA-mRNA theory proposed. However, any actual or potential dsRNA sequences have not been discovered in the mRNA preparations isolated (NAORA *et al.*, 1974). It is possible therefore that the dsRNA sequence is not inherited intact when the mRNA is being processed from the HnRNA. Indeed other sequences have been shown to be lost in the processing step. A ribonuclease T<sub>1</sub> resistant oligo-(U) sequence discovered by MOLLOY, THOMAS and DARNELL, (1972) and other regions derived from repeated sequences, including dsRNA, have been reported as not being inherited in the cytoplasmic mRNA (MOLLOY *et al.*, 1974).

If one looks at the structure of mRNA, it appears that in addition to the unique sequence coding for a particular polypeptide, and the poly(A) sequence, there appears to be a sequence which will hybridize to dsRNA and may be part of the dsRNA sequence derived from HnRNA. In rat liver mRNA, these sequences may constitute between 3 and 12% of the whole mRNA molecule (NAORA *et al.*, 1974) assuming that the length of cytoplasmic mRNA is 500-2000 nucleotides

(WILLIAMSON *et al.*, 1971; TAYLOR and SCHIMKE, 1973) and that the size of the dsRNA sequence is approximately 60 nucleotides. Sequences transcribed from repetitive sequences in DNA have been described in HnRNA (GREENBERG and PERRY, 1973; MOLLOY *et al.*, 1974 and HOLMES and BONNER, 1974). dsRNA has also been described as being transcribed from repetitive DNA sequences (HAREL and MONTAGNIER, 1971; JELINEK and DARNELL, 1972; RYSKOV *et al.*, 1973). Therefore the dsRNA in HnRNA is probably derived from repetitive sequences and the mRNA sequence which hybridizes to dsRNA from the HnRNA sequences is likely to be similarly derived from a repetitive DNA sequence. BISHOP *et al.*, (1972); HARRISON *et al.*, (1972) and SULLIVAN *et al.*, (1973) have indicated that the sequences coding for polypeptides are mainly transcribed from unique DNA sequences. It therefore appears that mRNA contains both repetitive and unique sequences. This view is supported by the recent finding of DINA *et al.*, (1973 and 1974), that *Xenopus laevis* embryos contain sequences transcribed from both unique and repetitive sequences. Possibly the sequences in *Xenopus* mRNA transcribed from repetitive DNA sequences are analagous to the sequences in rat liver mRNA derived from dsRNA. There is an apparent contradiction to this observation, in that sea urchin mRNA molecules appear to consist of almost exclusively unique sequence transcripts (GOLDBERG *et al.*, 1973; McCOLL and ARONSON, 1974). This discrepancy has not yet been resolved.

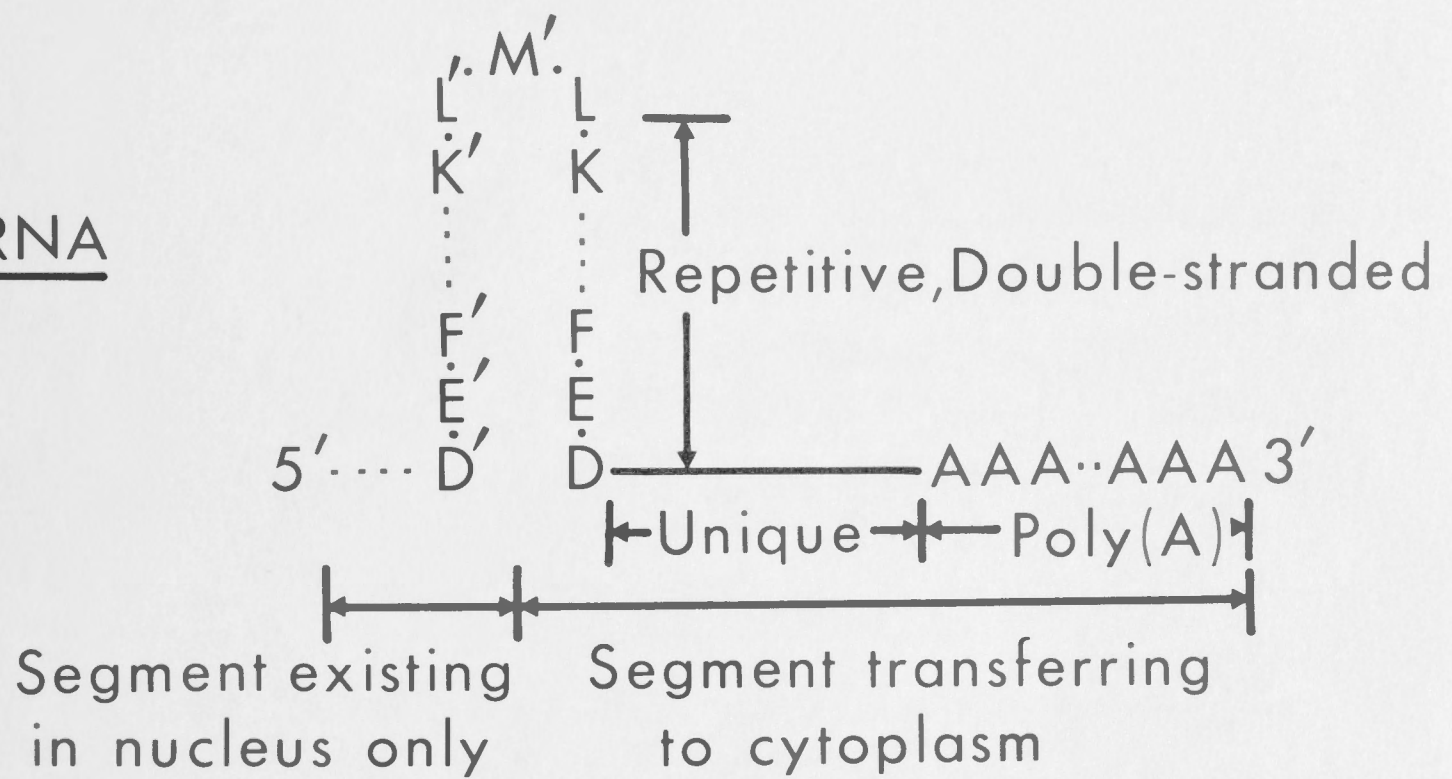


From the information about the nature of the mRNA molecule, and the fact that it contains a poly(A) sequence, a unique sequence and a region probably derived from part of dsRNA of a "hairpin" region, a model of mRNA and HnRNA can be constructed. This model is illustrated in Figure 7-2 and 7-3. It is known that the poly(A) segment occurs at the 3'OH end of the molecule (MENDECKI, LEE and BRAWERMAN, 1972; MOLLOY and DARNELL, 1973; and SHELDON *et al.*, 1972). The dsRNA derived segment would therefore need to be either between the poly(A) segment and the unique sequence, or at the 5' end. The latter possibility is more likely for the following reasons: (1) The apparent partial deletion of the whole dsRNA sequence is unlikely to have occurred within a molecule of mRNA. (2) The poly(A) segment is added irreversibly to the most of the HnRNA post-transcriptionally, but before the processing of mRNA began (DARNELL, JELINEK and MOLLOY, 1973; DARNELL, 1974). (3) No dsRNA segments were found covalently linked to poly(A) segments (see Chapter VI). (4) Sequences transcribed from repetitive DNA sequences have been detected at or near the potential 5' end of the HnRNA molecule (MOLLOY *et al.*, 1974). It is significant that repetitive sequences, 50-60 nucleotides long, have been found at the 5' end of *Xenopus* embryo mRNA (DINA *et al.*, 1974) which is about the length of the rat liver dsRNA obtained by gel electrophoresis.

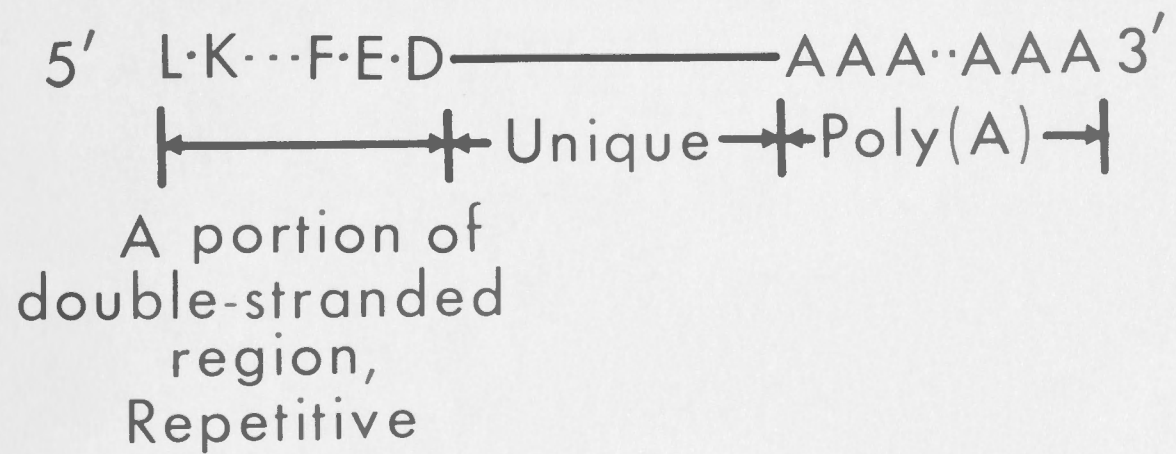
The model illustrated indicates a cytoplasmic mRNA

FIGURE 7-2    Schematic HnRNA and mRNA molecules

HnRNA



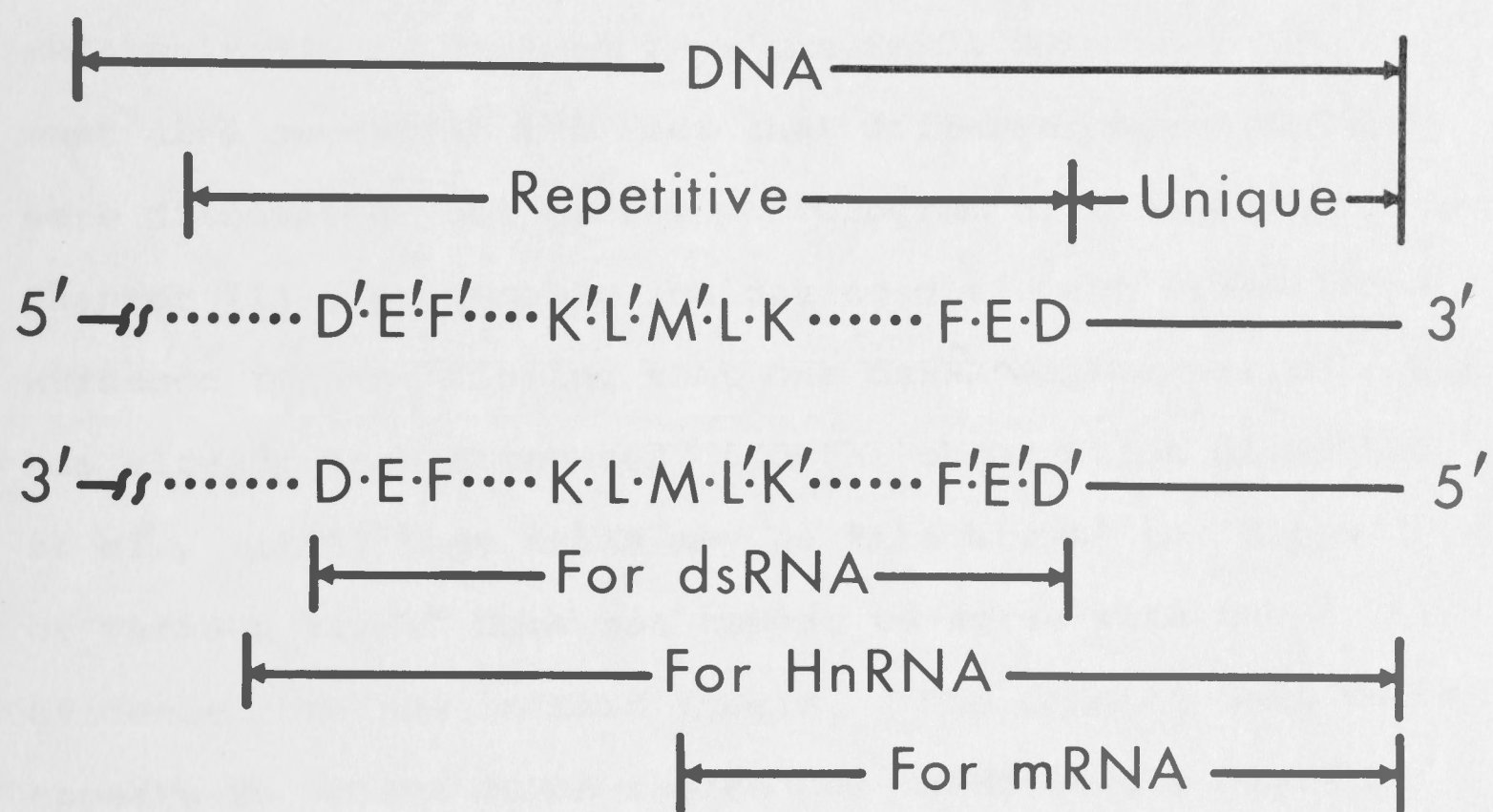
mRNA



Schematic HnRNA and mRNA Molecules



FIGURE 7-3    DNA sequences from which HnRNA, dsRNA and  
mRNA are transcribed



DNA sequences from which HnRNA, dsRNA  
and mRNA are transcribed

composed of a sequence corresponding to one strand of dsRNA at the 5' end, a unique sequence coding for the polypeptide and finally a poly(A) segment at the 3' end. The sequence at the 5' end of the mRNA molecule may be a residue of a double stranded region of the parent HnRNA molecule. It must be appreciated that the models may be oversimplified, since it is still unclear whether one mRNA molecule is derived from one HnRNA molecule. We must also reconcile the fact that different sized dsRNAs were discovered (gel electrophoretogram of size distribution Chapter III), presumably indicating different sized loops. Evidence for the finding that one dsRNA exists per molecule has already been discussed, and the observation of MOLLOY *et al.*, (1974) that dsRNA may be "the 'stems' to 'loops' of various sizes" does not appear to agree with the evidence obtained in this thesis. The finding that there appears to be one dsRNA region per HnRNA length could be regarded as evidence to support an idea of one mRNA derived from one HnRNA molecule.

The simplified model proposed is not inconsistent with the model of HnRNA suggested by MOLLOY *et al.*, (1974) and DARNELL, (1974). They propose, on the assumption that only one HnRNA yields one mRNA, that the HnRNA strand consists of running from 5' and 3' end, a sequence of oligo-(U) residues, a long section of repeated sequences interspersed with different sized dsRNA "hairpin" loops, then the unique sequence and finally the poly(A) segment



200 nucleotides long (Figure 7-1). As described above, the observations made for experiments in thesis suggested that only one dsRNA "hairpin" per molecule of HnRNA would exist and this is likely to be a close "hairpin" structure without a large connecting loop. This dsRNA loop would consequently occur in the region between the repeated sequence and the start of the unique sequence. Additional evidence indicates that the processing of HnRNA to mRNA is accomplished by specific enzymatic cleavage of HnRNA (LEE, MENDECKI and BRAVERMAN, 1971; GREENBERG and PERRY, 1972; FIRTEL, JACOBSON and LODISH, 1972 and JELINEK *et al.*, 1973). If a series of "processing" events occurred, the processing enzymes would probably require recognition sites common to the HnRNA molecule. It has recently been shown that oligo-(U) segments exist at the 5' end of the HnRNA molecule, these are apparently lost in progressively smaller HnRNA molecules and are certainly not found in mRNA (DARNELL, 1974). It is equally possible that the dsRNA "hairpin" region may also be a recognition site of a particular processing enzyme. This enzyme, if it exists, may recognise the loop end of the dsRNA "hairpin" and cleave the HnRNA molecule at that point. This explanation may account for the finding that half the dsRNA sequence is found in the cytoplasmic mRNA. No evidence for such a processing enzyme has been established but it is interesting to speculate that an enzyme may recognize a dsRNA region in a molecule. It is possible that dsRNA derived regions are involved in the recognition by messenger specific factors associated with ribosomes (HEYWOOD, 1970; NAORA,

KODAIRA and PRITCHARD, 1971) or in the post ribosomal supernatant (MATHEWS, 1970; NAORA and WHITELAM, 1972). In a later part of this discussion, evidence for specific recognition of dsRNA will be presented.

Whether or not exactly half the dsRNA sequences are inherited in the mRNA or whether any short segments of double stranded structure are retained in the mRNA, has yet to be thoroughly investigated. As mentioned already, it appears that there is no dsRNA in the mRNA isolated. However, the two following possibilities cannot be excluded. (1) A very small double stranded region in mRNA, or (2) only a few mRNA molecules, and hence only some of the HnRNA species have double stranded regions. Indeed it has been shown that both the protein coding and non coding portions of R17 and Q $\beta$  viral RNAs have double stranded character. In the protein coding region, loops of 20 base pairs were isolated and sequenced (STEITZ, 1969; ADAMS and CORY, 1970). It was predicted that mRNA in eukaryotes also contains dsRNA regions in the mRNA (WILLIAMSON *et al.*, 1971).

If only a portion of the double stranded sequence is retained, is the involvement of the sequence only in processing, or does it have some function in protecting a particularly valuable base sequence in the mRNA molecule, perhaps an initiator region? The function of the sequences at the 5' end of mRNA is not clear. It has been shown by other workers that eukaryotic mRNAs contain sequences which are not translated (WILLIAMSON *et al.*, 1971; SWAN *et al.*, 1972 and HOWELLS, 1973). In the case of insect mRNA, the initiator signal AUG

appears to be some distance from the 5' end (ILAN and ILAN, 1973). It is also possible that the portion of the dsRNA sequence in mRNA has no function and remains after HnRNA processing, or may have been involved in the actual transport across the nuclear membrane.

This discussion has raised a number of questions about the presence of a dsRNA species acting in the normal function of the cell. It is not possible to prove the actual existence of dsRNA in the '*in vivo*' state of the cell, and it could be questioned whether such dsRNA regions exist in this state. Nevertheless, the evidence for dsRNA as part of normal cellular function, seems overwhelming. The exact base sequence and whether or not it is translated remain unanswered questions. The discussion has assumed that all dsRNA in the cell is derived from HnRNA. This may indeed be true, but in the rest of the discussion other possible functions of dsRNA, whether of HnRNA, mRNA, or independent origin, will be considered.

#### 7-3-2 Interferon Induction

dsRNA in "uninfected" material was originally discovered by workers dealing with virus infected liver cells and in particular because of an interest in interferon. Interferon is an anti-viral agent found to be induced by different RNA and DNA viruses (HO *et al.*, 1966). Although the mechanism of interferon induction is not yet completely understood, it was speculated and shown that the double stranded replicative form of virus RNA was responsible for



induction of interferon (LAMPSON *et al.*, 1967; TYTELL *et al.*, 1967 and FIELD *et al.*, 1968). COLBY and CHAMBERLIN, (1969) and COLBY and DUESBERG, (1969) have further investigated this theory by studying induction in vaccina virus infected cells. More recently, STERN and FRIEDMAN, (1971) reported ribonuclease resistant RNA from chick liver to be a powerful interferon inducer in human fibroblast cultured cells. KIMBALL and DUESBERG, (1971) also reported that ribonuclease resistant RNA which appeared double stranded from several different cell cultures, was capable of inducing interferon.

These two latter reports, however, illustrate a new feature of the dsRNA induction of interferon. It appears that not only can a double stranded replicative form of a virus induce interferon, but also that a dsRNA fraction from an uninfected source can induce interferon. The amount of interferon induced may be quite significant. Indeed STERN and FRIEDMAN, (1971) reported induction three-fold greater than a well known inducer, synthetic dsRNA; poly(I).poly(C). The finding of induction in cells of the same species by dsRNA of homologous origin is highlighted in a report by DeMAEYER, DeMAEYER-GUIGNARD and MONTAGNIER, (1971) where they reported that the rat liver ribonuclease resistant fraction prepared by HAREL and MONTAGNIER, (1971) could induce interferon in rat embryo fibroblasts in culture. The amount of interferon induced in these cells appeared to be about the same as that induced by poly(I).poly(C) preparations. Furthermore, these workers were able to show that

the amount of interferon induced by the rat liver dsRNA exactly paralleled the heat denaturation enzyme susceptibility profile obtained with rat liver dsRNA.

These results however, do pose a serious question as to the actual induction of interferon. The hypothesis of interferon induction as proposed, states that induction is a response to a "foreign" source of RNA (ISAACS, 1961 and ISAACS *et al.*, 1963). It is difficult to disprove this theory, even in the light of the "uninfected dsRNA" action. It does appear however, that the double stranded nature of the RNA may be the only essential property for interferon induction. Even the results of DeMAEYER, DeMAEYER-GUIGNARD and MONTAGNIER, (1971) where both the dsRNA and induced cells were from the same animal species, the definition of "foreign" RNA could be restricted from the organism to the tissue level, where there may be differences. To finally demonstrate that the double stranded nature was the factor responsible, one would need to demonstrate that cellular dsRNA from an individual could induce interferon in cells derived from that same individual.

If the double stranded nature of DNA is involved in the mechanism of interferon, the following question can be raised immediately; why if resident cellular dsRNA universally exists in the cell, does it not induce detectable interference with virus growth? Perhaps the intra-cellular concentration of dsRNA is too low for interferon induction. It is difficult to

more of the dsRNA species which are not of dsRNA origin,

accurately estimate the percentage of free dsRNA in the cell, and it may be of cytoplasmic origin.

The experiments in this thesis did not detect significant quantities of dsRNA in the cytoplasm, and the amount of dsRNA in the whole cell, including the nucleus, was less than 1% of the total RNA. Most of this dsRNA appears to be part of HnRNA. The amount of free dsRNA capable of inducing interferon might therefore be as low as 0.01%. Since no significant quantities of dsRNA were detected in the cytoplasm, it is likely that the free dsRNA amount is less than 10% of the dsRNA and may be only 1%. The minimum amounts of poly(I).poly(C) necessary to produce interferon induction in chick embryo fibroblasts has been estimated to be  $10^{-8}$   $\mu\text{g}/\text{cell}$  (COLBY and CHAMBERLIN, 1969). The amount of RNA present in each rat liver cell is approximately  $5 \times 10^{-5}$   $\mu\text{g}/\text{cell}$  (CAMPBELL and KOSTERLITZ, 1952) and the amount of free dsRNA may consequently be  $5 \times 10^{-9}$   $\mu\text{g}/\text{cell}$  or less. This amount is at least 1/10th of the minimum amount of dsRNA required for interferon induction with poly(I).poly(C) and therefore induction in the normal cell may not occur. Higher levels of cellular dsRNA of the same source may still, however, be inducing.

What type of dsRNA is causing interferon production remains unsolved. It is difficult to imagine how the dsRNA species derived from HnRNA could be involved in an antiviral mechanism.

The possibility can be suggested therefore that cellular dsRNA is heterogeneous in origin and that one or more of the dsRNA species which are not of HnRNA origin,



are responsible for interferon induction. Each dsRNA species would then have a different function and experiments are certainly required to resolve which dsRNA species is involved in interferon production.

Another aspect of interferon production worth mentioning before turning to the action of interferon and dsRNA involvement in protein synthesis, is a possibility of using double stranded cellular RNA as an interferon inducer; where this might show less toxicity than the synthetic polynucleotides (poly(I).poly(C)), especially in the species from which it is derived. This may be a useful method of producing an anti-viral response.

The mechanism of interferon action will be briefly discussed here, because it reflects the apparent contradictions of the effects of dsRNA on protein synthesis. The action of interferon is predicted to be an interaction with the ribosome resulting in the formation of "interferon-type" ribosomes and ribosomal subunits which are capable of forming normal polysomes with host cell mRNA, but bind to viral RNA poorly, and are not able to translate it into protein (LEVY and CARTER, 1968). These workers demonstrated in the *in vivo* situation that "interferon-type" ribosomes were capable of forming polysomes with host cell mRNA, but would not form polysomes with the introduced viral RNA. In the "*in vitro*" situation, the purified ribosomes prepared from interferon-treated cells

- hereafter, interferon-treated ribosomes - were able to distinguish the host cell and viral messengers and translate only host cell mRNA. MARCUS and SALB, (1968) further suggested that interferon actually functions by derepressing the host genome to allow the production of a protein which they termed the translation inhibitory protein (TIP). The action of this was to interact with 40S ribosomal subunits and allow the ribosomes therefore to distinguish cellular from viral message, and secondly to block selectively translation of viral RNA.

dsRNA must therefore be acting by producing interferon, which in turn allows the selective translation of host mRNA, while blocking viral mRNA binding and translation. The exact details of the mechanism are still to be investigated but evidently dsRNA appears to be inhibiting viral protein synthesis but allowing cellular protein synthesis. There seems to be an apparent contradiction with this action of dsRNA and this will be discussed in the next section on dsRNA involvement in protein synthesis.

### 7-3-3 *dsRNA Involvement in Protein Synthesis*

In this section the possible direct involvement of cellular dsRNA in protein synthesis will be discussed. While investigating the initiation of protein synthesis in Hela cells by rabbit reticulocyte lysates, EHRENFELD and HUNT, (1971) discovered that initiation could be inhibited by polio virus. They indicated that this inhibitory action was due to the dsRNA form of the polio virus and that the

whole intact dsRNA molecule was not essential for inhibitory action. In a later communication, these workers described the detection of a complex between initiator tRNA (met tRNA<sub>F</sub>) and native ribosomal subunits in the crude reticulocyte lysate synthesizing globin at a high rate. The addition of dsRNA prepared from polio virus caused this complex to disappear. The formation of the complex appeared to be a natural event and the inhibitor was indeed dsRNA, since ribonuclease III, a nuclease specific for dsRNA digestion, (ROBERTSON, WEBSTER and ZINDER, 1967, 1968) abolished this inhibitory action. The other interesting feature noted by EHRENFELD and HUNT, (1972) was that only low concentrations (0.1-100 ng/ml) of dsRNA were inhibitory, whereas other polynucleotides, poly(I), poly(A), poly(U) only inhibited at high concentrations with different kinetics (HUNTER *et al.*, 1972).

Most recently the inhibition of protein synthesis by dsRNA has been reported as being caused by the inactivation of the initiation factor 1F-3, which is responsible for recognizing particular types of mRNA (KAEMPFER and KAUFMAN, 1973). The preparation of dsRNA employed was derived from virions associated with the fungus, *Penicillium Chrysogenum*. The protein synthesis system used was a rabbit reticulocyte lysate system, and it appeared that small amounts of dsRNA (as low as 0.1 ng/ml) were capable of causing firstly, decay of polysomes, an effect which was overcome by the addition of exogenous initiation factor 1F-3. It further appeared that dsRNA inactivated stoichiometric amounts of added 1F-3



and in fact formed a complex with this initiation factor, removing 1F-3 from the system. Without any added 1F-3, addition of dsRNA to the protein synthesizing system resulted in the inactivation of a vital initiation factor which existed originally, thus preventing the recycling of ribosomes and the binding to mRNA.

These findings suggest several further possibilities of dsRNA action. The specific inactivation of 1F-3 could be thought of as a possible mechanism of translational control in higher organisms. The residual protein synthesis observed in the presence of low levels of poliovirus dsRNA (EHRENFELD and HUNT, 1971) could be explained by the assumption that 1F-3 can be inactivated only when it is exchanging amongst ribosomal subunits after initiation of polypeptide synthesis, and that several rounds of exchange are needed to exhaust it completely. One dsRNA molecule could conceivably complex with a number of 1F-3 molecules, and thus affect a large number of ribosomes. This may explain the effectiveness of low concentrations of dsRNA.

The observations above suggest that 1F-3 may recognize double stranded regions in mRNA. It was found, however, that no dsRNA or potential dsRNA regions, were detected in mRNA. This was measured by the lack of ribonuclease resistant regions in the mRNA preparation (NAORA *et al.*, 1974). There is a slight possibility that very short dsRNA regions may be present in mRNA. JELINEK and DARNELL, (1972) report that 0.5% of mRNA is ribonuclease

resistant. Potential dsRNA sequences of at least 26 nucleotide pairs have been predicted in human  $\alpha$  globulin mRNA by the analysis of the amino acid sequence of the protein (LAUX, DENNIS and WHITE, 1973), but no direct evidence for dsRNA in mammalian mRNA has been discovered. In fact, in phage RNA, specific secondary structures have been observed for many segments of the single stranded chain, including ribosome binding and termination sites (STEITZ, 1969; BILLETER, 1969; GRALLA *et al.*, 1974). Although it is unknown how many nucleotide pairs in a dsRNA region the initiation factor 1F-3 could recognize, and in what form the *in vivo* molecular mRNA exists, the idea of specific recognition of a dsRNA region is worthy of further consideration.

There are criticisms which can be directed at the studies of KAEMFER and KAUFMAN, (1973). It must be mentioned that the inhibitory effect of dsRNA on protein synthesis was observed in an *in vitro* system. Furthermore, the dsRNA was prepared from a fungus, and the observed inhibitory effect may therefore be a tube phenomenon or a general reaction to "foreign" RNA. It might also be due to an undetected factor of viral consequence.

The biological significance of this phenomenon should not be underestimated in studying the functions of dsRNA. When cells are exposed to dsRNA it exhibits a dual function. (1) Interferon induction; and (2) inhibition of protein synthesis. dsRNA induces interferon in the host cell and

indirectly allows the translation of host specific mRNA, while blocking viral mRNA translation. dsRNA itself also inhibits protein synthesis by the formation of an 1F-3-dsRNA complex. This would in turn shut off cellular protein synthesis and not allow interferon or translation inhibitory protein to be produced. This may appear to be a contradiction of function, but there has been no evidence to suggest that these events compete in the manner suggested here. A plausible answer may lie in the effective doses of a particular dsRNA required to produce interferon or alternatively stop protein synthesis. The amount of dsRNA necessary for complete inhibition of protein synthesis varies enormously, depending on the source of dsRNA and the initiation factors involved. Of course, there may be an explanation as yet uncovered.

#### 7-3-4 *Possible dsRNA involvement in Self Duplication of mRNA*

There is another aspect of dsRNA involvement in normal cellular function which was hinted at in the Introduction, and which is the subject of a continuing investigation. This concerns the likelihood of self duplication of an RNA molecule.

In cells which are required for the synthesis of large quantities of a specific protein, large quantities of the specific mRNA molecule are needed. Normal synthesis of mRNA is from DNA, probably via HnRNA. Although more than



one copy of mRNA could either be synthesized by repeated transcription of a particular DNA sequence and accumulation of the mRNA product, or synthesis on repeated copies of the mRNA sequence in the DNA, it is attractive to propose that mRNA may be self-duplicated. This alternative route would not then be subject to the normal transcriptional or post-transcriptional controls, and would consequently enable the cell to switch on the synthesis of a specific protein.

The alternative requires an RNA-dependent RNA polymerase which recognizes mRNA as the template and produces an anti-mRNA copy from this template. The intermediate may be present as a double stranded form, or dsRNA may arise due to the association of + and - strands of the newly synthesized mRNA molecules.

The existence of a specific RNA-dependent RNA polymerase synthesizing an RNA molecule is then a reasonable indication that self-duplication may occur, particularly if the polymerase is specific for an mRNA template, and synthesizes a product which behaves as mRNA. The possibility of an RNA dependent RNA polymerase has been the subject of a recent investigation (NAORA and WHITEHAM, 1974). It was revealed that an enzyme system, i.e., RNA dependent RNA polymerase existed in normal rat liver cells. A crude enzyme fraction capable of catalyzing the polymerization of ribonucleotides into RNA, was prepared by

precipitation with ammonium sulphate at 50% saturation. The incorporation of labelled ribonucleotide tri phosphates catalyzed by the enzyme, is markedly stimulated by the addition of rat liver rRNA, but not by denatured or undenatured DNA. The reaction requires the presence of  $Mn^{++}$  (0.6 mM). The RNA directed RNA synthesis was not susceptible to actinomycin D, deoxyribonuclease or orthophosphate. The enzyme was also insensitive to  $\alpha$  amanatin and rifampicin. The former has been shown to be a potent inhibitor of eukaryotic nucleoplasmic DNA-dependent RNA polymerases, (JACOB *et al.*, 1970; KEDINGER *et al.*, 1970) and the latter an inhibitor of both bacterial and mitochondrial DNA-dependent RNA polymerases (WEHRLI and STAEHELIN, 1971; REID and PARSONS, 1971). Ribonucleases and pyrophosphate have been found, however, to strongly inhibit the reaction. These results suggest that the incorporation of ribonucleotides into RNA was apparently not due to polymerizing activities of either a polynucleotide phosphorylase or a DNA-dependent RNA polymerase, but probably due to the activity of an RNA-dependent RNA polymerase or a similar type of enzyme(s). Addition of synthetic polynucleotides poly(A), poly(U), poly(C) or poly(U,C) did not result in any significant stimulation of nucleotide incorporation. Purified 28S rRNA and tRNA produce only a small amount of stimulation. It should be mentioned here that rat liver dsRNA does show reasonable stimulation, but

the reaction appears to depend markedly on the presence or absence of high concentrations of 0.3M NaCl. With low salt concentrations in the assay mixture, almost no stimulation occurs with any dsRNA, i.e. poly(I).poly(C), reovirus dsRNA or rat liver dsRNA. The reason for this phenomenon is not clear at present, and further investigation is required to determine whether the effect of the salt is in maintaining the duplex structure of the dsRNA or altering the enzyme conformation, or both. As the products of the reaction were hybridized with the RNA added to the reaction mixture, the RNA added appeared to be a template rather than a primer. It is also interesting to note that the enzyme exhibited template specificity; the reaction by the rat liver enzyme(s) was stimulated in the presence of NaCl, by addition of rat liver dsRNA as well as nRNA. In contrast, no stimulatory activity was observed with reovirus dsRNA in the presence of NaCl.

These findings are especially significant if a dsRNA region exists at one end of the mRNA molecule. As already stated, the hybridization studies have found no evidence for a dsRNA region in mRNA, but it may only require a very short specific region for recognition by an RNA-dependent RNA polymerase. Such a short dsRNA sequence may exist in mRNA. RNA-dependent RNA polymerase might possibly recognize a short dsRNA region as the initiator sequence to begin the self duplication of the mRNA molecule. Small ribonuclease-resistant regions have been found in the



*in vitro* enzyme product, although this analysis is complicated by the difficulty of removing added template. The ribonuclease resistant complexes may represent the base paired regions associated with duplication.

*In vivo* RNA-dependent RNA synthesis has also been demonstrated with rat liver mRNA as a template. The stimulation observed was quite marked, in fact stimulation appeared to be much greater than when nRNA was added as a template. The nRNA fraction, however, contained other species of RNA, such as large quantities of precursors of rRNA and tRNA, as well as pre-mRNA, so no judgement can be made about the comparative effectiveness of mRNA or nRNA as a template. Both nRNA and mRNA are good templates for this enzyme. This finding is additional evidence to support the idea that mRNA may be undergoing amplification. A similar observation has been recently made, in that a reticulocyte RNA-dependent RNA polymerase, which uses globin mRNA as a template, has been discovered (DOWNEY *et al.*, 1973). The activities of this enzyme appear similar to that described by NAORA, (1974) and (WHITELAM and NAORA, unpublished observations.) No experiment has yet shown that the *in vitro* enzyme product is an entire (not partial) mRNA copy, or that it has the properties of an mRNA in stimulating active incorporation of amino-acids in a cell free system. Experiments to answer these queries are in progress. There is another problem also presently being investigated,

and that is the purification of the enzyme involved. It is hoped that purification of the enzymes will lead to more definite conclusions concerning the nature of RNA-dependent RNA synthesis.

The results obtained in the preliminary experiments have therefore indicated the possible role of dsRNA in this system in two ways, either as a specific site for initiation and/or recognition for mRNA amplification, or in the replicating complex itself. The other possibility is an artificial association between + and - mRNA strands synthesized. There is also one other possibility of dsRNA involvement linked with the observations of protein synthesis demonstrated by KAEMPFER and KAUFMAN, (1973). It has been shown by these workers that dsRNA inhibited the translation of protein synthesis. It could then be postulated that the newly synthesized dsRNA duplicating forms of mRNA, or the + and - mRNA strands associated as a double stranded complex, could inhibit the translational process by a mechanism similar to that demonstrated by KAEMPFER and KAUFMAN, (1973). The dsRNA complex may bind 1F-3 and prevent the formation of initiation complexes, allowing the preferential synthesis of RNA molecules by this alternative amplification process. Whether or not any of these involvements of dsRNA are feasible is still open to conjecture, but it appears likely that dsRNA may participate in a new process of amplification of the expression of specific genes, and the self duplication of the RNA message.

7-3-5 *Cell-to-Cell Information Transfer by dsRNA*

Possible functions of dsRNA in various reports would require lengthy discussion, and is beyond the scope of this thesis. The following section will only briefly mention one or two of the reports concerning dsRNA, in an attempt to emphasise that dsRNA may play an important role in transferring information from cell to cell. These reports are also mentioned here in the hope that they may be useful to further investigations on the functions of dsRNA. The concept of transfer of information proposes that genetic information passes from one cell to another. The molecular mechanism of information transfer is not exactly known, but nucleic acids, probably RNA, may be involved. Studies of the immune response have suggested that information concerning sensitization to a particular antigen may be passed from one cell to another, stimulating the receptor cell to blastogenesis and differentiation (FISHMAN, 1959). Further studies have revealed that the information is transferred in the form of "immunogenic" RNA. It has been thought that this RNA could transfer information to lymphocytes obtained from non-immunized animals and hence the lymphocytes would be capable of synthesizing and/or secreting immunoglobulins (FISHMAN, 1961; WILSON and WECKER, 1966; MOSIER and COHEN, 1968; WALKER, 1971). RNA appeared to be the macromolecule transferred during cell to cell contact (FISHMAN *et al.*, 1963). The argument against this observation



was that a trace amount of the antigenic material was associated with the "immunogenic" RNA and therefore the active component was not RNA, but the contaminating antigen. However, a recent report of "immunogenic" RNA has revealed that "immunogenic" RNA, completely free of antigens, is certainly active in transferring information and it may well be a dsRNA (ARCHER and WUST, 1973). These workers have reported that an "immunogenic" RNA, extracted by rigorous treatment with phenol-sodium dodecyl sulphate and pronase, is capable of transferring specific information from a population of sensitized lymphocytes to another unsensitized population. No antigen was found to be associated with this transfer. Of interest was that the "immunogenic" RNA was ribonuclease resistant and behaved like dsRNA. In particular, they indicated a biphasic heat-denaturation profile for their "immunogenic" RNA, which was paralleled by an irreversible loss of immunogenic activity. This latter point is strong evidence to suggest that dsRNA may be involved in transfer processes. Their report is preliminary and no particular weight can be given to this aspect of dsRNA involvement, but it is worthy of further consideration, and may lead to the understanding of information transfer between cells. From these findings, it is possible to conceive that RNA may in general play an important role in transferring cellular information, not only immunological information, but also

information associated with normal metabolism and cell function. It is attractive to propose that dsRNA may be the form in which RNA is transferred, especially when its ribonuclease resistant and chemically and physically stable base paired form is considered.

Other reports concerning information transfer by dsRNA included the finding of a new species of dsRNA in yeast (BERRY and BEVAN, 1972). The preliminary characterization of dsRNA from this source has revealed the presence of two high molecular weight species of dsRNA. From genetic evidence, these workers have predicted that the presence of the two dsRNAs determines the cytoplasmically inherited "killer" character observed in yeasts. The gel electrophoretic analysis reveals the two species to be in the 16S and 13S regions on polyacrylamide-formamide gels.

Such high molecular weight species have not been observed in experiments detailed in this thesis with uninfected rat tissues or other uninfected tissues, including cultured cells, examined by a large number of other workers (see Chapter I for details of references). Recently, however, DAVEY, (1973) reported a high molecular weight species of ribonuclease resistant RNA, which may be dsRNA, in "uninfected" mosquito cell cultures. The detailed nature of this material was never examined. In the case of the high molecular weight dsRNA species in yeast, a very recent report showed that the two dsRNA species are separately encapsulated into virus-like

particles (HERRING and BEVAN, 1974) similar to those described by ELLIS and KLEINSCHMIDT, (1969); BANKS *et al.*, (1969); BANKS *et al.*, (1970) and LLOAS, (1972), suggesting that these high molecular weight dsRNAs are of viral origin. A similar explanation may be the case for the results of DAVEY, (1973).

#### 7-3-6 Conclusion

In this discussion of the functions of the dsRNA species, an attempt has been made to illustrate the variety of fundamental processes in which dsRNA is likely to be involved. It is to be appreciated that in each case the dsRNA species has been studied from a different angle and with possibly vastly different isolation methods and examinations of function. Nevertheless, the physical and chemical properties of the dsRNA in each case are not different. The same basic characteristics peculiar to dsRNA can be used to identify the molecule. Methods of preparation and purification developed in this thesis can be universally applied to prepare dsRNA, and the characteristics used for identifying rat tissue dsRNA applied to positively identifying dsRNA as the functioning RNA species. While some of the experiments described in this thesis have been dedicated to the isolation, purification and characterization of dsRNA, the majority of the research has revealed a new approach to the investigation of the novel function of dsRNA, which has previously been obscure.



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